Use of Dried Plasma Spots for the Quantification of Iothalamate in Clinical Studies

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Summary
Background and objectives Although iothalamate clearances have been widely used to measure GFR, the need for transportation of plasma samples under refrigerated conditions obviates its use in resource-poor situations. Spots of blood or plasma dried on filter paper may provide a solution.

Design, setting, participants, & measurements Using a validated HPLC technique, iothalamate in dried blood spots of different hematocrits was measured. GFR was measured over 5 hours in 10 subjects with CKD using dried plasma spots and standard methods.

Results Lower hematocrit produced greater area of blood spreading and lowered the recovery of iothalamate from dried blood spots. However, the relationship between iothalamate concentrations in dried plasma spots and plasma showed a regression slope of 0.95 (95% confidence interval=0.92–0.98, P<0.001). Bland–Altman plot of paired sample points (n=116) showed a bias of −4 µg/ml and limits of agreement of −38 to +30 µg/ml. The relationship between GFRs using dried plasma spots and plasma methods also showed an excellent relationship (slope of 0.95, 95% confidence interval=0.82–1.17). Bland–Altman plot of paired GFRs showed a bias of 2 ml/min, with limits of agreement of −6 to +10 ml/min. Precision was generally between 5% and 10%, and accuracy was within 5%.

Conclusions Although dried blood spots are unsuitable for studies among those patients with very low hematocrit, dried plasma spots correct for this limitation, and this small pilot study shows that it is a reasonably reliable method for quantifying iothalamate and subsequently, determining GFR.


Introduction
GFR needs to be determined to accurately assess kidney function and stage CKD. To measure GFR, the clearance of inulin, a naturally occurring polysaccharide that is neither secreted nor reabsorbed by the renal tubule, is considered the gold standard (1,2). However, inulin is expensive and in short supply; therefore, alternative methods have been developed to measure GFR (3). One such method is to measure clearance of iothalamate (IOT) after bolus injection, providing an accurate and precise determination of GFR (4). Determining clearance of IOT through traditional plasma analysis does have limitations, including the need for venous cannulation for sampling of larger volumes of blood as well as the inherent cost of sample storage and transportation.

A possible solution to the above limitations is to use dried blood spots (DBSs) on a filter paper. Filter paper has been used for the collection, storage, and analysis of human blood since its adoption by Dr. Robert Guthrie for the detection of phenylalanine in phenylketonuria in neonates in the 1960s (5). Recent advances permit DBS to be increasingly used in clinical trials for the analysis of a number of small drug molecules (6–10). The advantages of DBS over plasma analysis include the following items. (1) Smaller blood volume (<100 µl) per sample makes the matrix applicable to pediatric or critically ill individuals, where larger blood volume may be undesirable. (2) Finger pricks can be used for sampling. This method of blood collection is easier to perform by staff, and it is preferred by subjects to venous cannula. Less invasive blood sampling can improve recruitment and retention of research participants. (3) DBS can be shipped and stored at ambient temperature, removing the necessity of costly freezers and transportation with dry ice. (4) The antimicrobial nature of the paper does not require biohazard arrangements during shipping (11). DBS does have its own limitations, primarily the uncertainty of the influence of blood hematocrit levels on test results.

Hematocrit may alter the viscosity of blood and may influence the results of quantification using DBS (12). For example, a high hematocrit blood sample is likely more viscous compared with a sample with a lower hematocrit level and may spread less on filter paper. In turn, it may vary the amount of sample obtained when a fixed diameter punch is taken from DBS. A solution to this vexing problem may be the use of a dried plasma spot (DPS), where viscosity is not dependent on the level of hematocrit. Accordingly, we hypothesized that (1) the hematocrit
will affect the amount of spreading on the filter paper and therefore, the plasma concentration of IOT, (2) DPS will correct for the hematocrit effect, and (3) DPS will provide an acceptable correlation with the reference method for determining GFR.

The DPS method would be most useful in populations where GFR estimations based on serum creatinine equations have not been adequate, such as those patients with liver cirrhosis or sarcopenia or patients with extremes of age. Serum creatinine equations are not optimal when comparing individuals across populations or among those patients who have minimal impairment of kidney function (13). DPS might be suitable in the above situations.

The purpose of this study is to describe the development and validation of a method for the quantification of IOT in human plasma, prepared as a DPS, detected through HPLC-tandem ultraviolet (UV). The method was validated through comparison of measured GFR using the traditional plasma analysis.

Materials and Methods

Chemicals, Reagents, Equipment, HPLC-UV

Instrumentation, and Method

Chemicals, reagents, equipment, and HPLC-UV instrumentation were established using a previously published method used to analyze standards and participant plasma samples (14). Additional chemicals, reagents, and equipment are as follows. Human packed red blood cells and fresh frozen plasma were provided through the Richard L. Roudebush Veterans Administration Medical Center blood bank (Indianapolis, IN). FTA DMPK-A, FTA DMPK-B, FTA DMPK-C, Harris 6-mm Uni-Core, and 903 Specimen Protein Saver Cards were obtained through GE Healthcare (Buckinghamshire, United Kingdom).

DBS Sample Preparation

Initial experiments suggested similar chromatography results with all filter papers. The 903 Specimen Protein Saver Cards were the least expensive; therefore, all experiments were performed using this paper. Using an adjustable micropipette, 15 μl blood were spotted onto the center of a 903 Specimen Protein Saver Card. Cards were allowed to dry in open air for 2 hours in a vertical position. Cards were then transferred to a desiccator containing loose dry desiccant for additional drying overnight. A 6-mm diameter disk was punched from the spot into a 2-ml microcentrifuge tube; 100 μl mobile phase (85% 50 mM sodium phosphate buffer, 10% methanol, 5% acetonitrile) were added. Samples were shaken for 60 minutes and then centrifuged for 2 minutes at 14,000 rpm. The resulting supernatant was transferred to a clean 1.5-ml microcentrifuge tube and centrifuged for 6 minutes at 14,000 rpm. The supernatant was then transferred to a clean vial, capped, and analyzed by HPLC-UV.

DPS Sample Preparation

Using an adjustable micropipette, 15 μl plasma were spotted onto the center of a 903 Specimen Protein Saver Card. The remainder of the DPS preparation follows the outline of the DBS samples.

Influence of Hematocrit Effect on DBS

Blood samples with defined hematocrit levels (21.2% or 33.5%) were produced by diluting packed red blood cells with fresh frozen plasma. For each hematocrit level, six DBS spots were made, digital images of the DBS were obtained, and the area of the blood spot was determined using the area calculation formula using Photoshop Creative Suite 5 (Adobe Systems, San Jose, CA). Full standard curves were prepared from each hematocrit concentration by spiking with IOT and spotted.

DPS Correction of Hematocrit Effect in Individuals

Plasma samples from 10 subjects containing a range of hematocrit (27.9±4.8) drawn at 12 points over 5 hours after bolus injection of IOT were spotted. Concentrations were compared with plasma samples not spotted on the filter paper and prepared in parallel. A Bland–Altman (mean difference) plot was used to assay the agreement between the DPS and traditional plasma methods.

Study Population

The study population consisted of 10 participants ranging in age from 41 to 82 years, with a mean (±SD) of 65.2±13.4 years. Six participants were men. Five participants were white, and five participants were black. Nine patients were mildly anemic, with a mean hemoglobin of 11.46±1.32 g/dl and a mean hematocrit of 34.04±4.19%. Mean estimated GFR was 33.4±10.1 ml/min per 1.73 m² (range=18–55.9 ml/min per 1.73 m²). The etiology of CKD was hypertensive nephrosclerosis in four participants, diabetes mellitus in four participants, and obstructive uropathy in two participants.

Comparison of DPS with Plasma in Estimating GFR

Pharmacokinetic parameters between parallel DPS and traditional plasma participant sample sets (n=10) were constructed. The GFR was estimated for each method and compared using the WinNonlin software (St. Louis, MO) and a two-pool pharmacokinetic model as previously described (15). Again, a Bland–Altman plot was used to assay the agreement between the two methods.

Method Validation: Inter- and Intraday Variability

The accuracy and precision of the method was determined by assaying replicates of full standard curves during the same run and on six separate occasions over 60 days. Accuracy and precision were determined. Precision was determined by calculating the coefficient of variation (CV) within 1 day (intraday precision) and between days (interday precision). Accuracy was determined by calculating the percent error (observed–predicted) ×100/predicted.

Plasma standard prepared to a concentration of one half the lowest point on the curve (25 μg/ml) was spotted onto 903 cards (n=14). Areas were calculated to determine detectability and precision. The protocol was approved by Indiana University Institutional Review Board for the protection of human subjects and the Veterans Affairs Research and Development Committee, and each subject signed a written informed consent.
Results

Influence of Hematocrit Effect on DBS
The area of spotted 33.5% hematocrit whole-blood samples was about 10% smaller than 22.2% hematocrit spots (Figure 1, Inset). Not surprisingly, the standard curve prepared with 22.2% and 33.5% hematocrit blood produced different spiked IOT peak area responses (Figure 1). As expected, compared with 22.2% samples, the mean slopes of the IOT area versus concentration in 33.5% hematocrit samples were 37% greater.

DPS Correction of Hematocrit Effect in Individuals
The relationship between participant IOT concentrations in DPS and plasma was excellent (Figure 2). Regression of the concentration estimated by the two methods showed a slope of 0.95, which was significantly different from 1. Thus, compared with the standard method of sampling IOT, the DPS method produced a mean paired sample point concentration that was 5% lower compared with plasma. A Bland–Altman plot of paired sample points (n=116) showed a bias of −4 μg/ml and limits of agreement of −38 to +30 μg/ml (Figure 2, Inset). Because the distribution of the differences was not normally distributed, resulting in a megaphone or V-shaped plot, we calculated the log-transformed differences. Bias was 1.7% (95% confidence interval=−22.3%, 21.0%).

Comparison of DPS with Plasma in Estimating GFR
The relationship between the estimated GFR in DPS and plasma methods was excellent (Figure 3). Although regression of the GFRs estimated by the two methods showed a slope of 0.95, it was not significantly different from 1, because the 95% confidence interval of the slope included 1. A Bland–Altman plot of paired GFRs showed a bias of 2 ml/min, with limits of agreement of −6 to +10 ml/min (Figure 3, Inset).

The paired plasma and DPS concentrate ions versus time for each of 10 subjects are shown in Figure 4. Most measurements differed little between methods shown quantitatively in Figure 2.

Test–Retest Accuracy and Precision
Test–retest assay performance is shown in Table 1. Precision was generally between 5% and 10%, and accuracy was within 5%. Statistically significant but small inaccuracies were seen for the highest and lowest IOT concentrations. Average CV and error for each standard curve point was within accepted criteria for assay validation (<15%) (15). The intraday slopes had a CV of 4.7%, whereas interday CV in slopes was 3.8%. We measured peak area with nominal concentration of 25 μg/ml IOT in plasma. In each instance, the IOT peak was clearly recognized, and the peak area had a CV of 9.7%.

We can be cautiously confident that IOT is stable in filter paper for 60 days. We prepared DPS samples on day 1 and ran these standards repeatedly to measure interday variability. The longest time that elapsed among IOT measurements was 60 days. At this time, there was good agreement in test–retest measurement as shown in Table 1.

Discussion
The results of our experiments show the development and validation of a simple, rapid assay to quantify IOT concentrations and subsequently, determine GFR in human DPS samples by HPLC-UV, showing an acceptable correlation.
Figure 2. The relationship between participant iothalamate concentrations using the standard method and dried plasma spot is shown. Regression of the concentration estimated by the two methods showed a slope of 0.95 with confidence interval of 0.92–0.98. Inset shows the Bland–Altman plot of paired sample points (n=116), which shows a bias of $-4\, \mu g/ml$ and limits of agreement of $-38$ to $+30\, \mu g/ml$.

Figure 3. The relationship between the estimated GFR using dried plasma spot and standard method is shown. Regression of the concentration estimated by the two methods showed a slope of 0.95 with confidence interval of 0.82–1.07. Inset shows the Bland–Altman plot of paired sample points (n=10), which shows a bias of 2 ml/min with limits of agreement of $-6$ to $+10$ ml/min.
with the traditional plasma processing. Several findings are noteworthy and will be discussed.

The change in hematocrit between samples corresponds to an observable change in the spot size of consistent volumes (9.8%). The greater spreading of samples with lower hematocrit causes a greater distribution of IOT on the filter paper. As expected, a fixed diameter punch taken from spots with differing hematocrit levels will effectively sample different volumes of blood. Thus, in samples with lower hematocrit, a lower IOT concentration is extracted. The change in peak area of IOT does not correspond linearly with an increase in the area of the spot and cannot be effectively compensated for in this assay. This finding is an especially important limitation of DBS when used in individuals with CKD who may have abnormally low levels of hematocrit. Although it is uncommon to measure IOT clearance to determine GFR in participants with severe anemia, compared with the DBS method, using the DPS method might yield more reliable results, especially among individuals with severe anemia.

Figure 4. Concordance between methods is shown as the time course of iothalamate decay curves over 300 minutes for each of 10 subjects. Each panel represents one subject; triangles denote the standard method, and the circles denote the DPS method.

<table>
<thead>
<tr>
<th>Table 1. Test–retest accuracy and precision</th>
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<tr>
<th>Nominal Iothalamate Concentration (μg/ml)</th>
<th>Mean Concentration (μg/ml)</th>
<th>SD</th>
<th>Precision (% Coefficient of Variation)</th>
<th>Accuracy (% Error, 95% Confidence Interval)</th>
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<tbody>
<tr>
<td>Intraday diagnostic test performance</td>
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<tr>
<td>50</td>
<td>48</td>
<td>3</td>
<td>6.3</td>
<td>2.4 (−11.3, 1.3)</td>
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<tr>
<td>100</td>
<td>96</td>
<td>7</td>
<td>6.9</td>
<td>2.7 (−10.9, 3)</td>
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<tr>
<td>200</td>
<td>194</td>
<td>26</td>
<td>13.3</td>
<td>5.3 (−16.5, 10.7)</td>
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<tr>
<td>300</td>
<td>300</td>
<td>20</td>
<td>6.5</td>
<td>2.7 (−6.8, 7)</td>
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<tr>
<td>500</td>
<td>511</td>
<td>16</td>
<td>3.2</td>
<td>1.3 (−1.1, 5.7)</td>
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<tr>
<td>1000</td>
<td>1052</td>
<td>53</td>
<td>5</td>
<td>2.2 (−0.3, 10.8)</td>
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<td>Interday diagnostic test performance</td>
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<tr>
<td>50</td>
<td>55</td>
<td>4</td>
<td>7.9</td>
<td>3.6 (1.3, 19.6)</td>
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<tr>
<td>100</td>
<td>103</td>
<td>8</td>
<td>7.9</td>
<td>3.4 (−5.2, 12)</td>
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<tr>
<td>200</td>
<td>212</td>
<td>18</td>
<td>8.3</td>
<td>3.6 (−3.3, 15.2)</td>
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<tr>
<td>300</td>
<td>311</td>
<td>27</td>
<td>8.8</td>
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<tr>
<td>500</td>
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<td>1.7 (−2.1, 6.6)</td>
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<tr>
<td>1000</td>
<td>1067</td>
<td>43</td>
<td>4</td>
<td>1.8 (2.2, 11.2)</td>
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The use of plasma on filter paper removes the variability of the hematocrit levels at the cost of a more complex sample preparation. Using DPS introduces the need of a centrifuge and blood collection tubes for fractioning whole blood. However, the sample preparation is still simpler than the traditional plasma method, maintaining a removal of the need for large volumes by venous cannula, storage, and sample transport limitations. The DPS provided a suitable alternative to plasma for quantifying the IOT concentration of plasma in CKD participants.

An important finding of our study was that, despite large variation in hematocrit levels among participants, no significant difference was observed in estimated IOT concentration between methods. The bias in the paired sample plasma versus DPS method was low, and the accuracy for each paired time point was within 15% between methods. Furthermore, the calculated GFR in participants provides accuracy within 15% between methods, showing that DPS is a suitable alternative to plasma for estimating GFR to assess renal hemodynamics and stage CKD.

All intra- and interday validation processes provided accuracy and precision within accepted criteria for assay validation and showed that analyte is stable in filter paper for up to 60 days (16). The peak areas of samples as low as 25 μg/ml in plasma could be easily visualized, and intraday test–retest reliability for peak area estimation was <10%.

Limitations of this pilot study include the small sample size of participants (n=10). Additional experiments involving larger samples would improve the statistical power of the tests. An additional limitation is that the presently used lower hematocrit level of 22.2% comparing spotting spread is at the lower limits of what may be seen clinically. A wide range of hematocrit levels is less likely to be encountered among stable individuals with CKD. Additional experiments involving narrower ranges of hematocrit levels might have be desirable to characterize the effect of hematocrit on DBS recovery.

Future experiments done to overcome the hematocrit effect could increase the field of application of DBS to include participants of varying hematocrit levels. Spotting a known volume and taking the whole spot for analysis would negate the effect of sample spread and provide analysis of a fixed volume of blood.

In summary, we show that DBSs are unsuitable for studies that include individuals with abnormal levels of hematocrit. However, DPSs correct for this limitation, and they are a reliable method for quantifying IOT and subsequently, determining GFR. The current method shows acceptable accuracy, precision, and robustness according to commonly accepted validation criteria. The DPS matrix may allow for sampling of populations that have previously been limited (pediatric or critically ill patients or those patients located in remote areas) because of lower blood volumes needed as well as provide a simpler, more economical means of storage and transportation of samples.

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Disclosures
R.A. serves on steering committees of trials funded by Roche, Amgen, Celgene, and Reata and speaker bureaus of Merck and Abbott and is a consultant for Daichii Sankyo, Inc., Takeda Pharma, and Sigma Tau.

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