tients treated with immunosuppressive agents after organ transplantation (5) and in patients with AIDS (15). Similarly, EBV-associated lymphomas that develop in immunosuppressed patients are suggested to be linked to the degree of competence of the immune system (16, 17). Clearly, such cases emphasize the role of immunosuppression in certain aspects of lymphomagenesis, irrespective of the cause of the immune defect. It has previously been suggested that EBV-associated epithelial and lymphoid malignancies involve the clonal expansion of a single EBV-infected progenitor cell (18, 19).

The mechanisms leading to the release of EBV DNA from tumor cells into the circulation are unclear. Several workers have suggested that the high concentrations of serum EBV DNA might be explained by increased EBV replication at sites other than the tumor (20), but such a proposition cannot account for the temporal changes and the rapid decrease in circulating EBV DNA in patients with NPC and lymphoid malignancies during the course of therapy (21). Recently it has been reported that short circulating DNA molecules are released into the circulation, possibly by apoptosis of cancer cells (22). In the present study, we compared the circulating EBV DNA in the blood compartments of patients with NPC and lymphoid malignancies by deriving an index, the cell-free percentage (%CF), to describe this dynamic phenomenon. A %CF value of 100% was found for all NPC cases, underscoring the exclusive existence of circulating cell-free EBV DNA in patients with this EBV-associated tumor. This is in sharp contrast to the very low %CF (0.2%) observed in lymphoid malignancies. The inclusion of %CF in future large-scale clinical studies on such EBV-associated disorders is thus warranted.

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References

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tocols have used zinc sulfate followed by acetonitrile (8), methanol (9), or acetone (10). Sometimes these reagents are premixed (11–14). During in-house assay development we encountered several problems when zinc sulfate and acetonitrile were used. For example, many specimens clumped when blood was added to the zinc sulfate. Even after addition of acetonitrile and vortex-mixing, the clumps remained, necessitating manual dislodgement of the pellet. This occurred with lysed human blood specimens, lyophilized whole-blood controls (e.g., Bio-Rad Lyphochek), and College of American Pathologists proficiency testing specimens. We substituted methanol for acetonitrile with minor improvement. Another problem was that the recovery of sirolimus from whole blood using zinc sulfate followed by acetonitrile or methanol was <100% (9). In addition, the observed MS/MS responses for commercial calibrators and controls were often ~30% higher than for human blood specimens.

Here we present a new extraction protocol that improves absolute recoveries, provides excellent precision, and shows less ion suppression. Although the performance of this protocol for several immunosuppressants is described, we present example data only for sirolimus. We also describe a solid-phase extraction (SPE) that may be added to further enhance the cleanliness of extracts.

For recovery and extraction experiments, we used cyclosporine from Novartis, tacrolimus from Fujisawa, and sirolimus from LC Laboratories. These same materials were used to prepare whole-blood calibrators, except for tacrolimus, for which Abbott Tacrolimus II calibrators were used. For precision studies we used Bio-Rad Lyphochek whole-blood controls.

The original zinc sulfate precipitation that we used was similar to previous publications. We added 50 µL of 0.1 mol/L aqueous zinc sulfate to a 1.5-mL polypropylene microcentrifuge tube. To this we added 50 µL of whole blood, followed by immediate vortex-mixing. We then added 500 µL of methanol containing 8 µg/L 32-desmethoxyrapamycin for sirolimus, 8 µg/L ascomycin for tacrolimus, or 20 µg/L cyclosporin D for cyclosporin A. After vortex-mixing for 30 s and centrifugation, the supernatant was analyzed.

In our new protocol we placed 50 µL of patient blood, calibrator, or control in a polypropylene microcentrifuge tube. To this we added, with no intermediate mixing, 250 µL of deionized water, followed by 250 µL of aqueous 0.1 mol/L zinc sulfate, and finally 500 µL methanol containing the appropriate internal standards. After addition of all components, the tube was vortex-mixed for 30 s. After 5–10 min the tubes were centrifuged for 4 min, and the colorless supernatant was analyzed.

SPE was performed with a 25-mg, 1-mL Varian LMS cross-linked styrene divinylbenzene column. The column was conditioned with 1 mL of methanol followed by 1 mL of water. The supernatant prepared above was passed slowly through the column (1–2 mL/min). The column was washed twice with 1 mL of water and air-dried under reduced pressure for ~30 s. The drugs were eluted into injection vials with 750 µL of acetonitrile. Before injection, 300 µL of water was added to each vial to make the solvent composition compatible with the initial mobile phase.

For HPLC-MS/MS analyses we used an Agilent 1100 binary system and a Waters Quattro Micro mass spectrometer. In the positive-ion mode the monitored multiple-reaction monitoring transitions (m/z) were: cyclosporin A, 1219.7→1202.7; cyclosporin D, 1233.8→1216.8; sirolimus 931.6→864.5; 32-desmethoxyrapamycin, 901.5→834.4; tacrolimus, 821.4→768.3; and ascomycin, 809.4→756.3. Separation was performed with a Phenomenex 4 × 3 mm (i.d.) C18 guard column maintained at 50 °C. The injection volume was 30 µL with a mobile phase flow rate of 0.4 mL/min. The mobile phases were as follows: (A), 2 mmol/L ammonium acetate and 1 mL/L formic acid in water; and (B), 2 mmol/L ammonium acetate and 1 mL/L formic acid in methanol. The gradient program was 50% B for 0.1 min, followed by an immediate change to 90% B at 0.11 min. At 1.8 min, the mobile phase was increased from 90% to 100% B to clean the column. At 2.3 min, the mobile phase reverted to 50% B. Sirolimus, tacrolimus, 32-desmethoxyrapamycin, and ascomycin eluted at ~1.6 min, whereas cyclosporin A and D eluted at ~1.8 min.

Extraction efficiencies were determined by enriching donor blood samples, stored at 4 °C, with sirolimus (10 and 20 µg/L), cyclosporine (100 µg/L), and tacrolimus (4 and 20 µg/L). Peak areas of the extracted samples were compared with areas obtained for injections of pure drug in saline (corrected for volume or dilution). After complete extraction, including SPE, recoveries were 95% for sirolimus, 95% for tacrolimus, and 103% for cyclosporine. These values are comparable to those reported by Deters et al. (7) and better than those reported by several other groups (6, 9, 11–14) for other procedures.

The improvements in absolute recovery from deidentified patient specimens, stored at 4 °C, obtained with our extraction protocol are shown in Fig. 1, panels A and B. Comparisons were performed within a single run to avoid day-to-day variation in instrument response. The increase in absolute signal for sirolimus, corrected for any dilution, averaged 36% with the new water hemolysis protocol. To evaluate whether the difference was attributable to ion suppression, specimens were also subjected to further SPE purification. After SPE, a similar increase of 39% in absolute recovery was observed, supporting the fact that, for sirolimus, the new protocol provided greater extraction efficiency. A similar increase in area response of 29% was observed for 32-desmethoxyrapamycin. A comparison of area response for cyclosporine and tacrolimus showed mean increases of 21% and 31%, respectively, with our water hemolysis extraction protocol. A comparison of the interrun CVs (Table 1) showed an improvement for sirolimus, tacrolimus, and cyclosporine. The limits of quantification (± 20%) were 0.5 µg/L for sirolimus, 0.2 µg/L for tacrolimus, and 5 µg/L for cyclosporine.

We evaluated ion suppression, using a 3 µL/min post-
column infusion of a methanolic solution of sirolimus that provided a signal similar to a specimen containing 3 μg/L drug (15). For a Lyphochek whole-blood control containing no sirolimus, the original extraction protocol showed ion suppression at several points in the chromatographic profile (Fig. 1C), whereas the water hemolysis protocol yielded cleaner extracts (Fig. 1D). Ion suppression was further reduced if SPE was performed (Fig. 1E).

During initial development, we became aware that some laboratories observed more consistent results for sirolimus if vortex-mixed tubes were allowed to sit before centrifugation. We included a 10-min incubation with our water hemolysis protocol, although we have not evaluated the necessity of this step.

There are several advantages to this new extraction protocol. One advantage is that the water helps lyse and uniformly dissolve blood components. Another advantage is that the use of water hemolysis and methanol as the solvent provides a desirable "milkshake" consistency after vortex-mixing. In addition, the extraction can be carried out with addition of all reagents followed by a single vortex-mixing step. Finally, this extraction could be adapted to an autodiluter for specimen delivery because

### Table 1. Interrun CVs.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration, μg/L</th>
<th>ZnSO₄/Methanol</th>
<th>H₂O/ZnSO₄/Methanol</th>
<th>H₂O/ZnSO₄/SPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sirolimus</td>
<td>10</td>
<td>8.6</td>
<td>4.4</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>7.6</td>
<td>4.3</td>
<td>4.2</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>9</td>
<td>4.6</td>
<td>2.4</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>2.7</td>
<td>1.7</td>
<td>1.4</td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>120</td>
<td>4.8</td>
<td>3.8</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>4.4</td>
<td>4.0</td>
<td>3.4</td>
</tr>
</tbody>
</table>

* Tacrolimus (n = 10), cyclosporine (n = 10), sirolimus (n = 12).
water allows the specimen to be flushed from the dis-

er can help restore the observed response for the
desired mass transitions.

References


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Diagnostic Accuracy of Cystatin C as a Marker of Kidney Disease in Patients with Multiple Myeloma: Calculated Glomerular Filtration Rate Formulas Are Equally Useful, Edmund J. Lamb, Helen J. Stowe, David E. Simpson, Anthony J. Coakley, David J. Newman, and Maeve Leahy (Departments of 1 Clinical Biochemistry, 2 Nuclear Medicine, and 3 Haematology, East Kent Hospitals National Health Service Trust, Kent and Canterbury Hospital, Canterbury, Kent, UK; 3 South West Thames Institute for Renal Research, St. Helier Hospital, Carshalton, Surrey, UK; * address correspondence to this author at: Department of Clinical Biochemistry, East Kent Hospitals NHS Trust, Kent and Canterbury Hospital, Canterbury, Kent CT1 3NG, UK; fax 44-01227-783077, e-mail edmund.lamb@ekht.nhs.uk)

Renal impairment is a common complication of multiple myeloma (1–3). Standard assessment of kidney function in myeloma patients includes serum creatinine and, in those found to have significant renal impairment, creatine clearance. This probably underestimates the prevalence of kidney disease. The availability of an improved measure of kidney function would aid in the selection of chemotherapy, improve monitoring of kidney function during bisphosphonate treatment, enable detection of kidney disease at an earlier stage, and improve avoidance of potentially nephrotoxic drugs.

The limitations of serum creatinine as a marker of the glomerular filtration rate (GFR) are widely appreciated (4,5). Creatinine clearance may be more sensitive, but it requires a timed urine collection, which is imprecise (6) and inconvenient (7). For clinical purposes, 51Cr-labeled EDTA clearance provides a surrogate gold standard measure of GFR (6,8), but it is time-consuming, expensive, and not readily available in many hospitals.

Attempts to improve clinical measurement of GFR include the use of creatinine-based formulas, including those proposed by Cockcroft and Gault (9) and Levey and coworkers [Modification of Diet in Renal Disease (MDRD) formula (10) and its simplified version (11)]. These improve GFR estimation compared with serum creatinine alone (12), although considerable limitations persist. Cystatin C is a 13-kDa protein whose plasma concentration reflects GFR. Its superiority over serum creatinine in terms of diagnostic sensitivity for reduced GFR is generally accepted (13–21), but concerns have been expressed that it may be affected by malignant progression (22–26). To our knowledge, neither cystatin C nor the MDRD formulas have been evaluated against a gold standard measure of GFR in patients with multiple myeloma.

For this study, we recruited 39 Caucasian volunteers with a confirmed diagnosis of multiple myeloma through hematology outpatient clinics. The study was approved by the Local Research Ethics Committee. Patients were prospectively enrolled from April 2001 to February 2003 during times the investigators were available. Exclusion criteria were active rheumatoid disease, renal dialysis, or renal transplantation. Patients attended the hospital for a 51Cr-EDTA clearance within 1 month of recruitment,