in lot 5 ($P = 0.001$, Fisher exact test), whereas the methadone false-positive rate was 4% in lot 2 and 19% in lot 5 ($P < 0.001$, Fisher exact test). The between-lot discrepancies in false-positive rates between assays on the same device reduced the likelihood that changes in operator behavior over time (either intra- or interoperator) explain the lot-to-lot variability observed. In addition, each device was reviewed the next day by a single technologist, with <1% of results being changed on review, which also limited the likelihood that operator error was an important contributor to interlot variability. However, the fact that different urine samples were analyzed on each lot of the lateral-flow device must be recognized as a limitation of our study and prevents us from ruling out operator variability as an important contributor to our results.

Lot-to-lot variability was also observed in false-negative rates across the 5 lots. In particular, lot 4 showed an oxycodone false-negative rate 5-fold higher than the mean false-negative rate for the other 4 lots (15% vs 3%, $P < 0.001$). This led to a drop in negative predictive value from a mean of 96% to 77%.

We also found that the cross-reactivity of specific analytes in specific assays on the lateral-flow device showed substantial lot-to-lot variability. This is best illustrated by hydrocodone, which cross-reacts with the oxycodone immunoassay per the package insert and leads to 56% of the oxycodone false-positive results (from hydrocodone measured by LC-MS/MS). However, detectable hydrocodone (>10 ng/mL) caused a false-positive oxycodone result 90% of the time in all lots but lot 4 (only 60%).

To mitigate the cost of testing to patients, we have designed a risk-based reflexive strategy. Non-compliant lateral-flow screening results are confirmed with LC-MS/MS (2). As a result of this strategy, we find the lot-to-lot variations in the false-positive and false-negative rates acceptable. However, laboratories need to be aware of these issues and may need to consistently rely on confirmatory testing to properly assess compliance in high-risk patients.

Note: The Clinical Chemistry editorial office invited American Bio Medica Corporation to submit a Reply to this letter but received no response.

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Engineered Human Lipocalin as an Antibody Mimetic: Application to Analysis of the Small Peptide Hormone Hepcidin

To the Editor:

Anticalin® proteins are human lipocalins that are engineered to bind relevant targets with high affinity and specificity (1). By applying a mutation and selection process, it is possible to select Anticalin proteins that can specifically bind to very diverse protein targets in a monovalent fashion. Anticalin proteins are also suited for engaging small and compact ligands owing to their cuplike binding pocket. Compared with monoclonal antibodies, Anticalin proteins are about 8 times smaller and can be recombinantly produced in bacterial cells in large amounts.

The hepatic peptide hormone hepcidin is a highly conserved molecule of only 25 amino acids with 4 disulfide bridges that plays a central role in body iron metabolism (2). Therefore, it could become a useful biomarker. Numerous hepcidin assays, by use of either mass spectrometry (MS)-based1 techniques or traditional immunochemical assays,
have been described to quantify hepcidin in biological fluids (2). However, both MS and immunochemical approaches have disadvantages, including costly and sophisticated instrumentation, low throughput, need for highly specialized personnel, and difficulties in obtaining high-quality antibodies. The latter is ascribed to the small size of hepcidin, its folded nature, and its conservation throughout evolution, which complicate the generation of hepcidin antibodies for immunochemical assays such as ELISA. Thus, there is a need and opportunity for alternative approaches.

We developed a competitive bioanalytical assay for plasma and serum hepcidin using the Anticalin protein Hep-Dx as a next-generation diagnostic antibody mimic and biotin-labeled hepcidin-25 as a tracer. Hep-Dx is closely related to another highly specific and potent hepcidin antagonizing Anticalin protein that was developed for therapeutic purposes and that exhibited an affinity of 50 pmol/L for hepcidin-25 and its amino-terminal truncated isoforms including hepcidin-20 (3).

We began by coating microtiter plates with 100 μL/well Hep-Dx (80 μg/L in coating buffer, 15 mmol/L Na₂CO₃, and 35 mmol/L NaHCO₃, pH 9.6) overnight at 4 °C, followed by blocking for 30 min with Superblock (Thermo Scientific) at ambient temperature. The hepcidin-25 calibrator (4392-s, Peptides International), unknown samples, and internal control samples (75 μL/well in dilution buffer of PBS with 1% BSA and 0.1% Tween-20) (4) were added, followed by 75 μL/well biotinylated hepcidin (obtained by 30-min incubation of hepcidin-25 with No-Weigh™ Sulfo-NHS-Biotin (Pierce Chemical), dilution 10 000×). After incubation overnight at 4 °C, the plates were incubated for 1 h with 100 μL/well streptavidin–β-peroxidase conjugate (dilution 50 000) and subsequently with 100 μL substrate solution (4 mg ortho-phenylenediamine in 10 mL borate/citrate buffer) for 15 min in the dark. The color reaction was stopped with 100 μL of 0.5 nmol/L H₂SO₄, and absorbance (492 nm) was measured.

The dose–response curve of the calibrator hepcidin-25 was approximated by a 4-parameter logistic curve and showed a dynamic range (SD) of 21 (8) pmol/L to 3.1 (1.2) nmol/L (n = 24). All measurements were performed in duplicate. The analytical lower limit of detection (LLOD) was defined as the minimum hepcidin concentration evoking a response statistically (95% CI) different from that of the zero calibrator and was 21 pmol/L. The functional LLOD was calculated from the imprecision profile (at 20%) constructed from 231 individual duplicate CVs and was 23 pmol/L.

Intraassay (n = 8) CVs of the low (3.5 nmol/L), intermediate (6.0 nmol/L), and high (16.0 nmol/L) control samples were 7.0%, 6.3%, and 5.9%, respectively, and interassay (n = 14) CVs were 8.9%, 6.9%, and 10.3%. Assay specificity tests by constructing dose–response curves
for synthetic hepcidin-20, -22, -24, and -25 showed that the Anticalin-based assay detected hepcidin-25, as well as all 3 smaller isoforms, virtually indistinguishably. Three serum samples containing native hepcidin-25 (7.0, 17.3, and 63.2 nmol/L) were serially diluted 5–80× in dilution buffer. In each sample, the dilution curve was close to linear, confirming parallelism between the calibrator and the samples. When we added 3 concentrations of synthetic hepcidin-25 to 6 serum samples (endogenous hepcidin-25, 0.6–2.5 nmol/L), the mean recovery was 93.4% (range 69.7%–109.7%). Finally, hepcidin concentrations for 28 plasma/serum samples obtained by the Anticalin assay were compared with values found by our previously validated weak cation exchange time-of-flight MS assay (4) and a competitive ELISA (5) for which reference ranges have been described (2). Absolute hepcidin concentrations were similar for the 3 assays (Fig. 1).

In summary, our competitive Anticalin-based hepcidin assay eliminates the use of expensive antibodies that are often difficult to obtain in case of small and conserved peptides, and it correlates with existing assays for hepcidin. Anticalin-based assays could be useful for studying concentrations of small and highly conserved peptides, among which is hepcidin. This assay demonstrates the successful use of scaffold binding proteins other than antibodies in diagnostic medicine.

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