without extraction. Furthermore, sample extraction further improved the accuracy of the second generation assays in the low range (Table 1; also see online Supplemental Data Fig. 1), which indicates that they still suffer from interfering compounds and their specificity can be further improved. For several immunoassays an increased bias was observed (Table 1); this finding could be explained by the design of the assay, which is not calibrated for a purified reconstitution matrix.

The phenomenon that direct automated testosterone immunoassays give inaccurate high results in samples from women and children because of possible calibration and specificity problems has been addressed extensively in the literature. The precise nature of the interfering compounds has yet to be elucidated, although a significant cross-reactivity with DHEA sulfate has been reported for the Cobas® I and II assays (5).

In the present study we evaluated the accuracy before and after sample extraction of 7 commercially available direct testosterone assays. In conclusion, in the >4.0 nmol/L testosterone range, all immunoassays showed a good correlation with ID-LC-MS/MS. The correlation coefficients for automated immunoassays were relatively poor for measurement of low testosterone concentrations (<4.0 nmol/L) in untreated samples. Following diethyl ether sample extraction, the correlation coefficient of 6 of 7 immunoassays, including the second generation assays, increased. Although the accuracy of testosterone immunoassays has been improved since 2007 by the introduction of second generation assays has been improved since 2007 (Table 1); this finding could be explained by the design of the assay, which is not calibrated for a purified reconstitution matrix.

In the present study we evaluated the accuracy before and after sample extraction of 7 commercially available direct testosterone assays. In conclusion, in the >4.0 nmol/L testosterone range, all immunoassays showed a good correlation with ID-LC-MS/MS. The correlation coefficients for automated immunoassays were relatively poor for measurement of low testosterone concentrations (<4.0 nmol/L) in untreated samples. Following diethyl ether sample extraction, the correlation coefficient of 6 of 7 immunoassays, including the second generation assays, increased. Although the accuracy of testosterone immunoassays has been improved since 2007 by the introduction of second generation assays, there is still a need for further improvement.

References


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To the Editor:

Measurements of urine or plasma metanephrine (MN)1 and normetanephrine (NMN) are considered pivotal tests in the diagnosis of pheochromocytoma and paraganglioma. There has also been increasing interest in measuring 3-methoxytyramine (3MT), the corresponding O-methylated metabolite of dopamine. 3MT measurement might allow earlier diagnosis of rare cases of pheochromocytoma and paraganglioma that secrete predominantly dopamine (1–3). 3MT measurement might also enable stratification of familial cases by secretion patterns and play a role in predicting the malignant potential of pheochromocytoma and paraganglioma (2).

1 Nonstandard abbreviations: MN, metanephrine; NMN, normetanephrine; 3MT, 3-methoxytyramine; HPLC-ECD, HPLC–electrochemical detection; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MN-d₃, deuterium-labeled MN.
Eisenhofer and others have used HPLC–electrochemical detection (HPLC-ECD) to measure 3MT in plasma (1–4). de Jong et al. developed an online solid-phase extraction liquid chromatography–tandem mass spectrometry (LC-MS/MS) method that simultaneously measures NMN, MN, and 3MT (5). We attempted to reproduce the latter method for plasma 3MT measurement and were initially pleased with its analytical performance. When we attempted to establish a reference interval for a healthy population, however, it became apparent that our upper reference limit of 73 pg/mL (0.437 nmol/L) was much higher than the 1–14 pg/mL (0.006–0.084 nmol/L) interval for the published HPLC-ECD method (1). de Jong et al. also observed a higher upper reference value in their study (5).

We determined subsequently that there is substantial cross talk between MN and 3MT ion pairs in our single reaction monitoring LC-MS/MS method. Calibration material containing MN and NMN, but not 3MT, was found to produce a measurable 3MT ion peak, although the ion pairs do not appear related (m/z: 3MT, 151.1/91.1; MN, 180.1/148.1; NMN, 166.1/134.1). The MN contribution to the 3MT peak was nearly 2% of the injected MN concentration. In a patient sample with a relatively high MN concentration, this amount of cross talk will lead to overestimation of the normally much lower endogenous 3MT concentration (Fig. 1, left). Unfortunately, ionic cross talk also impedes the use of the less-intense qualifier ion pair (m/z 151.1/119.1).

We explored this phenomenon further by performing product ion scans of the 180 m/z ion during infusion of 10 μg/mL MN in 700 mL/L methanol at a flow rate of 10 μL/min. This experiment revealed the predicted dominant product ions at m/z 165 and m/z 148, as well as a product ion of m/z 151—which corresponded to the 3MT precursor ion—at an approximately 10-fold lower intensity. During this infusion, a product ion scan of m/z 151 showed a typical 3MT fragment of m/z 91, and a precursor ion scan of the m/z 91 ion gave precursors of m/z 151 and m/z 180. We also observed a similar fragmentation pattern with the deuterium-labeled MN (MN-d3) internal standard, NMN, and the NMN-d3 internal standard, but to a lesser extent.

This discovery suggested that MN and, to a lesser extent, MN-d3, NMN, and NMN-d3 had the ability to fragment within the source into ions mimicking 3MT. The MN, NMN, and 3MT precursor ions usually represent a hydroxyl loss and the addition
of a charged H\(^+\). A proportion of MN, MN-d\(_3\), NMN, and NMN-d\(_3\) might also lose methyl or hydroxyl groups to give 3MT precursor ions. Considering the formulae of MN, NMN, and 3MT and a range of different ionization conditions, variable proportions of MN or NMN (or their internal standards) might be “converted” to a 3MT mimic.

On the basis of these findings, we attempted to optimize source conditions to reduce this cross talk but were unable to strike a balance between reduced cross talk and required analytical sensitivity. Chromatographic separation of MN and 3MT, which was absent in the initial method, proved necessary to resolve this issue. We found that ion-pairing liquid chromatography using 1.25 mmol/L perfluoropentanoic acid as the ion-pairing agent (gradient from 100 to 400 mL/L methanol; flow rate, 0.5 mL/min) and an Agilent Zorbax Eclipse XDB-C18 column (4.6 \(\times\) 50 mm, 3.5 \(\mu\)m) for separation resolved 3MT from MN effectively, achieving a 60-s separation between the 2 peaks (Fig. 1, right). Not surprisingly, a peak corresponding to the monitored 3MT ion pair could still be seen at the MN retention time, further emphasizing the importance of peak resolution.

Our work suggests that cross talk needs to be addressed and evaluated in any mass spectrometry assay, and if identified, appropriate actions should be taken to minimize cross talk interferences through chromatographic resolution. We found that whenever MN, NMN, and 3MT are not resolved chromatographically, as in the case of our original method and in the method of de Jong et al., there is the risk of MN or NMN contributing to the 3MT peak. Given that the concentrations of both MN and NMN in plasma are much higher than those of 3MT, that will lead to falsely high 3MT measurements.

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**References**


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**Binding of Hepcidin to Plasma Proteins**

**To the Editor:**

The hepcidin hormone of 25 amino acid residues is a key regulator of iron homeostasis (1). Hepcidin has been shown to bind in vitro to \(\alpha_2\)-macroglobulin (\(\alpha_2\)M)\(^1\) and albumin in human plasma (2). It is not known, however, to what extent hepcidin is bound to proteins in vivo. For purposes of clinical interpretation, it is critical to know whether protein-bound or free hepcidin is being quantified.

To characterize the binding of hepcidin to plasma proteins, we used gel filtration to fractionate 0.5 mL serum samples from both healthy individuals and patients undergoing hematopoietic stem cell transplantation. We similarly fractionated mixtures of hepcidin and \(\alpha_2\)M or albumin [1 g/L in 10 mmol/L potassium phosphate buffer, pH 7.4, containing 150 mmol/L NaCl (PBS)]. We used a Superdex™ 200 10/300 GL column (GE Healthcare Biosciences) equilibrated with PBS at a flow rate 0.5 mL/min. The column was calibrated with synthetic hepcidin (Peptide Institute), human albumin, and \(\alpha_2\)M. We collected 0.5-mL fractions and measured hepcidin in the fractions by HPLC–tandem mass spectrometry (LC-MS/MS) as previously described (3). We observed a single peak, corresponding to free hepcidin, eluting at 21–25 mL. In patient

\(^1\) Nonstandard abbreviations: \(\alpha_2\)M, \(\alpha_2\)-macroglobulin; LC-MS/MS, HPLC–tandem mass spectrometry.