

Stability of Urinary Fractionated Metanephrines and Catecholamines during Collection, Shipment, and Storage of Samples

JACQUES J. WILLEMSSEN,¹ H. ALEC ROSS,^{1,2*} JACQUES W.M. LENDERS,³ and FRED C.G.J. SWEEP¹

Background: Measurements of 24-h fractionated urinary metanephrines and catecholamines are used for the diagnosis of pheochromocytoma, but adequate information is needed regarding collection, storage, and shipment conditions.

Methods: Spot urine samples were collected from 8 healthy volunteers. Aliquots were immediately frozen at -20°C , or acidified to pH 4 and then frozen either directly or after 24 h at room temperature. The remaining urine was left at room temperature for 24 h and then split into one portion that was acidified and one portion that was not. Aliquots were either frozen or allowed to stand at room temperature for an additional 24, 48, 72, 96, and 168 h before freezing. We also tested the efficacy of adding Na_2EDTA and $\text{Na}_2\text{S}_2\text{O}_5$, as an alternative to acidification for preservation of the catecholamines.

Results: No clinically relevant degradation ($<5\%$) was observed for the fractionated metanephrines under any of the storage conditions. In contrast, in $\sim 50\%$ of the untreated samples catecholamines were partially degraded during the first 24 h at room temperature. Immediate acidification, however, prevented degradation, whereas acidification after 24 h prevented further decay. Addition of Na_2EDTA and $\text{Na}_2\text{S}_2\text{O}_5$ fully prevented degradation of catecholamines during the first 24 h in 4 of 5 cases. In the remaining case, degradation did not exceed 10%.

Conclusion: Preservation of samples for measurements of urinary fractionated metanephrines is not necessary if samples are assayed or frozen within 1 week, which is an important advantage if transport of samples is nec-

essary. In contrast, urinary catecholamines require preservation measures during collection.

© 2007 American Association for Clinical Chemistry

Pheochromocytomas are rare neuroendocrine, catecholamine-producing tumors that arise from the chromaffin cells of the adrenal medulla or from sympathetic ganglia (paraganglioma). Excessive production of the catecholamines epinephrine (EPI)⁴ and norepinephrine (NE) and their metabolites metanephrine (MN) and normetanephrine (NMN) is considered a hallmark in the biochemical diagnosis of catecholamine-producing tumors. Although there is evidence that plasma concentrations of the free (unconjugated) metanephrines MN and NMN are better indices than other manifestations of catecholamine excess for detecting pheochromocytomas (1–4), measurements of fractionated metanephrines and catecholamines in urine are still commonly used for the diagnosis of pheochromocytoma. These assays are infrequently requested; so many laboratories prefer to send samples to more specialized reference facilities. Shipment of frozen samples on dry ice is considered the most secure mode of transport for urinary and plasma catecholamines, but has a number of obvious drawbacks. Various authors have reported on the stability of catecholamines in urine and the use of alternatives to hydrochloric acid (5–12) for urine preservation, as well as on the stability of catecholamines in heparin plasma (12), but only a few studies have reported stability studies of metanephrines in urine (13, 14) and plasma (15). Chan reported on the degradation of free (unconjugated) MN and NMN and catecholamines in pooled urine samples and deconjugation of their sulfoconjugates (13). Moleman used Na_2EDTA and $\text{Na}_2\text{S}_2\text{O}_5$ as preservatives and stored the urine samples at -20°C immediately after collection (14). Because available data are partly contradictory (5, 7, 12, 13) or scarce, we studied the stability

¹ Departments of Chemical Endocrinology, ² Endocrinology, and ³ General Internal Medicine, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands.

* Address correspondence to this author at: 479 Dept. of Chemical Endocrinology, Radboud University Nijmegen Medical Center, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands, Fax 31-24-3541484; e-mail a.ross@ace.umcn.nl.

Received June 21, 2006; accepted November 2, 2006.

Previously published online at DOI: 10.1373/clinchem.2006.075218

⁴ Nonstandard abbreviations: EPI, epinephrine; NE, norepinephrine; MN, metanephrine; NMN, normetanephrine.

of urinary (fractionated) metanephrines and catecholamines at room temperature, both during and up to 1 week after the first 24 h of collection, with and without preservative measures (i.e., acidifying to pH 4 or addition of Na₂EDTA and Na₂S₂O₅ during collection and acidification to pH 4 after collection of 24-h urine portions).

Materials and Methods

STUDY PARTICIPANTS AND SAMPLING

After obtaining informed consent, we collected spot urine samples from 4 male and 4 female volunteers. These samples were subdivided and processed according to a scheme that resulted, after 8 days, in sets of 1.2-mL aliquots with the following characteristics:

- immediately frozen at -20°C .
- immediately acidified to pH 4 and frozen
- immediately acidified to pH 4 and frozen after 24 h
- left for 24 h at room temperature and then frozen, or frozen after a subsequent period of 24, 48, 72, 96, or 168 h of storage at room temperature without preservation
- left for 24 h at room temperature, acidified to pH 4 and then frozen, or frozen after a subsequent period of 24, 48, 72, 96, or 168 h of storage at room temperature in an acidified condition

With this protocol we were able to test the effects on the stability of both metanephrines and catecholamines in samples that were left standing at room temperature for up to 8 days (d vs a or b) and acidifying during (c vs a or b) or after the first 24 h at room temperature (e vs a or b) and (e vs d).

The results of these experiments led to an additional study in which spot urine samples were collected from another 5 healthy volunteers (2 males and 3 females) for catecholamine assay. Each urine sample was divided into 2 equal parts, and to one part, as an alternative preservation method, Na₂EDTA and Na₂S₂O₅ (25 mg of each) were added immediately after collection.

CHEMICALS AND REAGENTS

All reagents were of analytical grade and MilliQ reagent water was used. NE, EPI, internal standard isoproterenol, MN, and NMN were obtained from Sigma. Na₂EDTA and Na₂S₂O₅ were obtained from Merck. For measurements of the catecholamines and metanephrines, we used the same reagents and materials as previously described (16, 17), but the reagent set from Bio-Rad Laboratories (17) has been replaced by a newer version.

INSTRUMENTATION

An Alliance Separations Module Model 2695 (Waters Associates) was used for HPLC measurements of the catecholamines and metanephrines in urine. Chromatographic data were processed with Empower Pro Workgroup, including Oracle9i® database Software version 1154. Separation and detection were performed as previously described (16, 17).

SAMPLE PREPARATION

Urine samples for measurement of the catecholamines were processed as previously described (16), with the following exceptions: 25 μL of urine were added to 1 mL 0.01 mol/L HCl before liquid-liquid extraction, which was performed only once. We made the final extraction in 75 μL of 0.08 mol/L acetic acid. To convert the catecholamines into their diphenyl-quinoxalin derivatives, we used twice the amounts of the solutions. After derivatization, 25 μL of the solution was injected into the chromatographic system. Processing of the urine samples for measurement of metanephrines was as described (17), without modification.

SENSITIVITY, PRECISION AND ACCURACY

Analytical characteristics of assay performance have been described (16, 17). Most relevant to the present experiments were the within-run CVs for measurements of the catecholamines and metanephrines, which were calculated from 24 duplicate measurements of the 1st sample of each volunteer. These were 1.8% for NE, 1.9% for EPI, 2.9% for NMN, and 2.4% for MN.

CALCULATIONS

We used Empower Pro Workgroup, including Oracle9i® database Software package version 1154 from Waters Associates for HPLC-FD and HPLC-ECD peak analysis. With each series of samples, a standard mixture was analyzed. Peak-height ratios for each analyte relative to the corresponding internal standard isoproterenol (catecholamines) or 4-O-methyldopamine (metanephrines) were determined in both the standard mixture and the urine samples. These ratios were used to calculate the concentrations in the urine samples.

STATISTICS

We calculated 1-sided 95% confidence intervals from the within-run CVs for percentage of baseline concentrations. Degradation was assumed to have taken place from the point in time that only values below the confidence limits were observed. Degradation was considered relevant when it exceeded 10%.

Results

For the unpreserved urine samples, 3 of 8 urine samples showed degradation for NE (-12% , -28% , and -13%) during the first 24 h after voiding and storage at room temperature, and 4 of 8 samples showed degradation for EPI (-10% – -40%) (Table 1). When these urine samples were acidified to pH 4 immediately after voiding, no degradation of the catecholamines was found (Table 1). Moreover, acidification prevented decay in one sample that suffered from degradation (F2; NE -18% and EPI -24%) when stored frozen without acidification (not shown). In contrast, no degradation of fractionated metanephrines was found in any of the 8 unpreserved urine

Table 1. Effect of storage for 24 h at room temperature (rt) compared with storage at -20°C , with and without acidifying to pH 4, in 4 different samples in which EPI and/or NE degradation was observed after 24 h at room temperature.

Study participant	NE		EPI	
	24 h rt as percent of 24 h, -20°C	24 h rt pH 4 as percent of 24 h, -20°C pH 4	24 h rt as percent of 24 h, -20°C	24 h rt pH 4 as percent of 24 h, -20°C pH 4
F2	88.0	98.5	71.6	100.5
M2	95.0	99.7	89.5	99.2
M3	72.4	101.3	60.3	100.5
M4	86.7	99.6	74.3	98.5

samples, irrespective of acidification, including the 4 in which catecholamine decay occurred (Table 2).

After the first 24 h at room temperature (equivalent to a usual collection period), further degradation of the catecholamines was observed during the next 7 days in the same 4 unpreserved urine samples (Fig. 1). The decreases ranged from -25% to -78% for NE and -39% to -89% for EPI. Moreover, in 2 of the other unpreserved urine samples a moderate decrease was found after 96 h (NE -6% and EPI -11%) and after 192 h (NE -5% and EPI -11%). There were only 2 unpreserved urine samples in which no degradation was found after storage at room temperature during the study period. In contrast, acidification to pH 4 after the first 24 h at room temperature prevented further decay (Fig. 1).

No degradation of fractionated metanephrines was found in any of the unpreserved urine samples for the next 7-day period at room temperature (Fig. 1, Table 2).

When the stability of the catecholamines was examined with and without the addition of Na_2EDTA and $\text{Na}_2\text{S}_2\text{O}_5$ immediately after voiding, there was a slight decrease of NE (-5.4%) and EPI (-6.2%) in 1 of 5 unpreserved urine samples, and during the first 24 h at room temperature a slight decrease of only EPI (-8.9%) in a preserved urine sample. After 4 days, a protective effect attributable to the addition of Na_2EDTA and $\text{Na}_2\text{S}_2\text{O}_5$ became evident. In 1 unpreserved sample, both NE and EPI had virtually disappeared, whereas the decay was limited, although not sufficiently, to -14% for NE and -19% for EPI when the additions had been made. After 8 days, NE and EPI had practically disappeared from an additional unpreserved urine sample, whereas the maximal change in the pre-

served urine samples was -25.8% for NE and -43.5% for EPI.

Discussion

Although assays of urinary fractionated metanephrines and catecholamines are more widely available than those of plasma metanephrines, even these assays are performed only in more specialized centers, and samples must often be shipped to these centers. Therefore, insight into the stability of these analytes during collection, storage, and shipment is indispensable. Giles and Meggiorini (5) reported deterioration of catecholamines at room temperature in pooled samples. Miki and Sudo (7) reported on the stability of catecholamines during the first 24 h of collection, but their study included samples from only 2 volunteers. Iu et al. (10) reported on stability of catecholamines, but did not include information about the first 24 h. Elfering et al. (9) and Gouarne et al. (11) observed deterioration of catecholamines during the first 24 h at room temperature in their study of the stability of the catecholamines in untreated urine samples collected from 9 or 10 volunteers. The contrasting results from the studies of Miki and Sudo (7) and Boomsma et al. (12), indicating that unpreserved urine samples are suitable for the measurement of the catecholamines, were obtained in pooled urine samples.

In our experiment 8 volunteers participated, and we purposely did not pool the obtained urine samples. We found degradation of the catecholamines in unpreserved urine samples after the first 24 h at room temperature in 3 of 8 cases for NE and in 4 of 8 cases for EPI. In contrast, no degradation was found in 4 of the other urine samples

Table 2. Mean urinary concentrations of metanephrines as a percentage of initial value, measured at 6 time intervals in 8 different samples after collection and storage at room temperature, with and without acidification 24 h after collection.

Days at room temperature	1	2	3	4	5	8
Unpreserved MN (SD), % of initial value	100.4 (3.6)	98.0 (3.2)	98.9 (4.8)	97.1 (4.6)	96.9 (4.1)	99.6 (8.3)
Range	97.0–108.4	91.3–101.6	94.3–109.0	92.7–107.5	88.4–101.6	94.4–119.7
pH 4 MN (SD), % of initial value	97.1 (4.1)	96.2 (2.3)	93.9 (3.4)	97.7 (4.7)	97.1 (3.8)	97.0 (6.3)
Range	93.1–105.5	93.1–99.3	88.4–98.4	92.9–108.4	92.5–103.7	90.5–109.7
Unpreserved NMN (SD), % of initial value	99.8 (2.2)	96.8 (4.9)	97.2 (4.4)	98.3 (4.4)	98.7 (5.0)	99.6 (5.7)
Range	96.7–102.2	88.7–102.2	89.0–101.5	91.3–104.7	93.3–108.8	89.6–107.2
pH 4 NMN (SD), % of initial value	97.4 (3.3)	98.4 (4.4)	97.2 (3.6)	97.2 (6.8)	96.8 (7.5)	98.5 (5.6)
Range	91.6–101.3	92.7–105.9	89.7–101.6	84.0–105.3	83.4–106.5	87.9–105.2

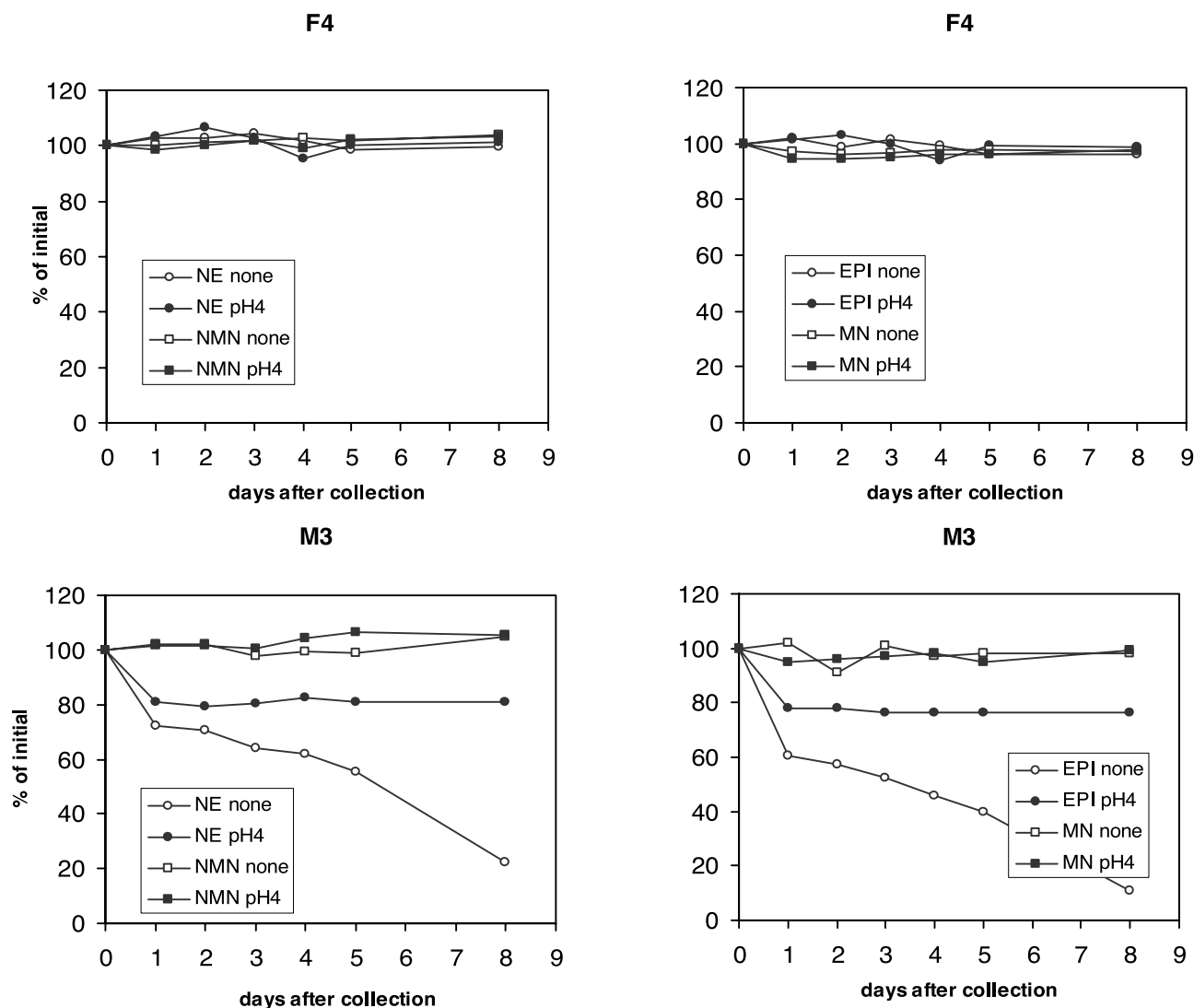


Fig. 1. Two representative patterns of catecholamine and metanephrine concentrations in urine during storage at room temperature for up to 8 days and the effect of acidifying to pH 4 after 24 h.

No detectable decay, as in depicted female study participant F4, was encountered in females F1 and F3, and the male M1. Male study participants M2 and M4 and female F2 displayed a pattern similar to the depicted male M3.

after the first 72 h of storage at room temperature, and only a small decrease was found in 1 unpreserved urine sample after 96 h storage at room temperature (NE –6% and EPI –11%) and in another urine sample after 192 h storage at room temperature (NE –5% and EPI –11%). In 2 urine samples no degradation was found even after 192 h of storage at room temperature. Apparently, catecholamine decay may vary widely between individual samples, even for samples from healthy persons, ranging from no deterioration to complete degradation of the analytes. Therefore, inclusion of too few individuals, or the use of pooled material, may yield misleading results, an observation that may explain the seeming contradiction between our results and those of Miki and Sudo (7) and Boomsma (12). For stability studies in general, we advise against reliance on pooled urine, serum, or plasma.

The cause of this variability (initial pH, microbial growth, proportion of unconjugated analyte) may be the subject of further study.

As confirmed by earlier observations (5–12), acidifying to pH 4 is an effective means to prevent catecholamine decay, even when it is not done immediately after voiding (Fig. 1). For those urine samples in which degradation starts during the first 24 h and without acidification continues at room temperature for the next 7 days, acidification after the first 24 h abolishes further degradation. Chan et al. (13) reported that unpreserved pooled urine samples stored at –80 °C were stable, with <10% variation for at least 22 days. In one of our cases, we observed degradation during storage at –20 °C in the unpreserved but not in the acidified sample. To achieve acidification in practice, HCl may be added in advance to

the polyethylene collection container, but this leads to hydrolysis and poses a hazard to the patient when the acid is still concentrated (7, 10, 13) during the first phase of collection. The catecholamine concentrations found under such conditions result from the opposing effects of deconjugation and decay. Moreover, insufficient antioxidative capacity may lead to catecholamine instability. Therefore, addition of Na₂EDTA and Na₂S₂O₅ instead of HCl during collection, followed by acidification to pH 4.0 with HCl after receiving the urine sample is mandatory. Boric acid (6) or formate buffer (10) also can be used for this purpose.

The special requirements to stabilize catecholamines during collection and storage at room temperature do not appear to be necessary for fractionated metanephrines in urine (Table 2), at least for a 1-week period. Nevertheless, acidification to pH 4 immediately after receipt of the 24-h urine sample can be considered as a general safety measure. We did not find data in the literature allowing for a direct comparison with the present observations. Our findings are highly relevant to the biochemical diagnosis of pheochromocytoma according to recommendations emerging from the First International Symposium on Pheochromocytoma (2005, Bethesda, MD). First, concentrated acid need not be added to the devices used for urine collection; thus the risk for the patient of injury by the acid is eliminated. Second, if samples are assayed or frozen for assay within 1 week, urinary metanephrines are sufficiently stable at room temperature to permit shipment by regular mail. Third, the absence of additives may permit measurement of other analytes such as urinary sodium and potassium, which are essential to the interpretation of aldosterone excretion when assessed in the same sample, as well as albumin and total protein.

We thank Renate Hensen-Lodewijk, Jannette Beunk, Marie-José Leupers, and Rob van den Berg for their helpful cooperation.

References

1. Lenders JW, Pacak K, Walther MM, Linehan WM, Mannelli M, Friberg P, et al. Biochemical diagnosis of pheochromocytoma: which test is best? *JAMA* 2002;287:1427–34.
2. Raber W, Raffesberg W, Bischof M, Scheuba C, Niederle B, Gasic S, et al. Diagnostic efficacy of unconjugated plasma metanephrines for the detection of pheochromocytoma. *Arch Intern Med* 2000;160:2957–63.
3. Eisenhofer G. Biochemical diagnosis of pheochromocytoma: is it time to switch to plasma free metanephrines? *J Clin Endocrinol Metab* 2003;88:550–2.
4. Peaston RT, Weinkove C. Measurement of catecholamines and their metabolites. *Ann Clin Biochem* 2004;41:17–38.
5. Giles HG, Meggiorini S. Stability of catecholamines in urine [Letter]. *Clin Chem* 1983;29:595.
6. Lee ZSK, Critchley JAJH. Simultaneous measurement of catecholamines and kallikrein in urine using boric acid preservative. *Clin Chim Acta* 1998;276:89–102.
7. Miki K, Sudo A. Effect of urine pH, storage time, and temperature on stability of catecholamines, cortisol and creatinine. *Clin Chem* 1998;44:1759–62.
8. Weinkove C. Measurement of catecholamines and their metabolites in urine. *J Clin Pathol* 1991;44:269–75.
9. Elfering A, Grebner S, Semmer NK, Byland C, Gerber H. Two urinary catecholamine measurement indices for applied stress research: effects of time and temperature until freezing. *Hum Factors* 2003;45:563–74.
10. Lu YP, Ho CS, Mak TWL. Formate buffer as preservative for urinary free catecholamine measurement. *Ann Clin Biochem* 2004;41:39–42.
11. Gouarne C, Foury A, Duclos M. Critical study of common conditions of storage of glucocorticoids and catecholamines in 24-h urine collected during resting and exercising conditions. *Clin Chim Acta* 2004;348:207–14.
12. Boomsma F, Alberts G, van Eijk L, Man in 't Veld AJ, Schalekamp MA. Optimal collection and storage conditions for catecholamine measurements in human plasma and urine. *Clin Chem* 1993;39:2503–8.
13. Chan ECY, Wee PY, Ho PC. Evaluation of degradation of urinary catecholamines and metanephrines and deconjugation of their sulfoconjugates using stability-indicating reversed-phase ion-pair HPLC with electrochemical detection. *J Pharm Biomed Anal* 2000;22:515–26.
14. Moleman P. Preservation of urine samples for assay of catecholamines and their metabolites [Letter]. *Clin Chem* 1985;31:653–4.
15. Willemsen JJ, Sweep CGJ, Lenders JWM, Ross HA. Stability of plasma free metanephrines during collection and storage as assessed by an optimized HPLC method with electrochemical detection. *Clin Chem* 2003;49:1951–3.
16. Willemsen JJ, Ross HA, Jacobs MC, Lenders JWM, Thien T, Swinkels LM, et al. Highly sensitive and specific HPLC with fluorometric detection for determination of plasma epinephrine and norepinephrine applied to kinetic studies in humans. *Clin Chem* 1995;41:1455–60.
17. Willemsen JJ, Ross HA, Wolthers BG, Sweep CGJ, Kema IP. Evaluation of specific high-performance liquid-chromatographic determinations of urinary metanephrine and normetanephrine by comparison with isotope dilution mass spectrometry. *Ann Clin Biochem* 2001;38:722–30.