Clin Chem Lab Med 2009;47(6):786–790 © 2009 by Walter de Gruyter • Berlin • New York. DOI 10.1515/CCLM.2009.162

Letter to the Editor

Proficiency test of plasma free and total metanephrines: report from a study group

Dilo Pillai[1], H. Alec Ross[2], Jürgen Kratzsch[3], William Pedrosa[4], Ido Kema[5], Kirsten Hoad[6], Nathalie Rouaix[7], Marc Fathi[8], Herbert Nader[9], Bruno Mathian[10] and Eric Grouzmann[11,*]

[1] Department of Clinical Chemistry, The Prince of Wales Hospital, Randwick, Australia
[2] Department of Chemical Endocrinology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands
[3] Institute of Laboratory Medicine, Clinical Chemistry and Molecular Diagnostics, University Hospital Leipzig, Leipzig, Germany
[4] Instituto Hermes Pardini Aimorés, Aimorés, Brazil
[5] Department of Clinical Chemistry/Binding Analysis, University Medical Centre Groningen, Groningen, The Netherlands
[6] Core Clinical Pathology and Biochemistry Pathwest Laboratory Medicine WA Royal Perth Hospital, Western Australia, Australia
[7] Centre Hospitalier Régional Universitaire de Lille, Laboratoire de Biochimie, Lille, France
[8] Service de médecine de laboratoire, Hôpital Cantonal Universitaire de Genève, Genève, Switzerland
[9] RECIPE GmbH, Munich, Germany
[10] ProBioQual, Lyon, France
[11] Division of Pharmacology and Clinical Toxicology, University Hospital of Lausanne, Lausanne, Switzerland

Keywords: pilot study; plasma free metanephrines; plasma total metanephrines; proficiency testing.

The diagnosis of pheochromocytoma is based on the quantitation of O-methylated metabolites of catecholamines (1, 2). Several studies have reported high sensitivity of free metanephrines in plasma for assessing the presence of tumour (1). Measurement of plasma total metanephrines (plasma free+plasma sulpho-conjugated metanephrines) has also been proposed as a diagnostically sensitive assay (3). Therefore, an increasing number of laboratories have developed assays for free and total metanephrines using different analytical procedures including immunoassays (4, 5), high-performance liquid chromatography (HPLC) coupled to amperometric or coulometric detection (6) and liquid chromatography-tandem mass spectrometry (LC-MS) methods (7). Each laboratory is supposed to establish its own reference values and to perform quality control measurements (QCs) with each set of analyses. The internal QC samples usually are prepared by each laboratory and should include plasma samples spiked with known concentrations of both metanephrine (MN) and normetanephrine (NMN). Participation in an external quality assessment scheme (EQAS) allows direct comparisons between all laboratories that measure free and total metanephrines. In this context, the variability of the upper reference limit for free metanephrines reported in the literature is relatively large. For NMN the range varies from 0.6 to 1.4 nmol/L, and for MN it varies from 0.3 to 0.85 nmol/L (1, 2, 8). However, the upper reference limits for total metanephrines are better defined (3, 9, 10). The analytical procedure for the quantitation of metanephrines in plasma is not the only reason for this variability. The characteristics of the control population may also differ between different studies. The absence of a universal calibrator that could be used by all laboratories performing such measurements encouraged us to establish an EQA program for plasma free and total metanephrines. A similar program already exists for metanephrines and catecholamines in urine. The purpose of this letter is to present the results of a pilot study aimed at implementing a proficiency survey for all laboratories measuring plasma free and/or total metanephrines.

We selected 10 centres that routinely measure free and/or total plasma metanephrines for diagnosis of pheochromocytoma. Three analytical methods were used. Immunoassays (1 participant), HPLC with electrochemical detection (ECD) (8 participants) including amperometry, coulometry, coularray and LC-MS (1 participant). All but one laboratory, which used an RIA commercial kit method, used in-house methods. Calibrators and internal quality control samples were manufactured using spiked serum pools with standard dilutions of Sigma D, L MN and NMN. The survey provided one result for free metanephrines in plasma by radioimmunoassay (RIA), one by LC-MS and five by LC-ECD while sulpho-conjugated metanephrines gave one result by LC-MS and four by LC-ECD. Two types of matrices were compared. Although heparinised plasma is normally used by the majority

*Corresponding author: Dr. Eric Grouzmann, PhD, Division de Pharmacologie et Toxicologie Cliniques, University Hospital of Lausanne, Lausanne, Switzerland
Phone: +41-21-3140741, Fax: +41-21-3144266,
E-mail: eric.grouzmann@chuv.ch

of the laboratories, this matrix was not used since it forms aggregates during lyophilisation of the samples and may affect analysis. Therefore, one laboratory involved in this study (HPLC with coularray detection, Eric Grouzmann) compared heparinised and citrated plasma samples collected from three different subjects; the concentrations of free metanephrines found in the two matrices were similar. In addition, the chromatographic profiles were clean in both sample types (data not shown). Therefore, we decided to use serum and citrated plasma as an alternative matrix to heparin. Serum (samples 1 and 2) and citrate plasma (samples 3 and 4) were prepared by RECIPE (RECIPE CHEMICALS & INSTRUMENTS GmbH, Munich, Germany), a company that produces quality control materials as lyophylisates to be reconstituted in water. The citrate-plasma (CPD-plasma) that we used for preparing the EQA-samples was prepared from an aliquot of a pool from 36 individuals (pool volume in total: ~10 L). Serum was used from a pool prepared from more than 100 individuals. The samples contained normal, but unknown concentrations of NMN and MN. A second set of samples (samples 2 and 4) were spiked with exogenous NMN (956 pmol/L) and MN (456 pmol/L) to mimic concentrations observed in plasma of a patient with pheochromocytoma. Since sulpho-conjugated metanephrines standards were not available for spiking experiments, we were unable to assess their recovery. Methods used for hydrolysing sulpho-conjugated metanephrines included incubation of the sample with sulphatase or, alternatively, perchloric acid precipitation of proteins from serum, followed by boiling.

For the immunological method, the recovery was only 50% of the added amount of NMN and MN. This is because the antibodies used in the RIA are stereoselective and only measure the physiological form of metanephrines, while the exogenous spiking material contains both the D and L forms of NMN and MN. Therefore, results obtained for samples spiked with NMN and MN, using immunoassay methods, were corrected to compensate for D isomers that were not measured as follows: corrected concentration of spiked samples = 2 × concentration of spiked sample–concentration of unspiked sample. The lyophilisates were shipped to PROBIOQUAL, a non-profit French centre that organises external quality surveys for laboratories. The data were directly sent to PROBIOQUAL who provided the survey results to the study coordinator (Eric Grouzmann). The number of participants in this pilot study was too low to allow for extensive statistical evaluation of results. Therefore, data were compared on the basis of means and recoveries of spiked metanephrines, and we only compared the overall recoveries of metanephrines added in plasma and serum by mean of a two-way ANOVA. Inter- and intra-method coefficients of variation observed between all the results were also reported. The dispersion of concentrations reported for free NMN and MN was relatively small between laboratories. Percent CVs ranged from 10% to 34%. The results for MN from laboratory #3 were excluded since they were very high (probably HPLC interference). Percent CVs were similar for serum and plasma free NMN and MN (Table 1). Mean concentrations of free NMN from citrate plasma were different, although in the same range, compared to those measured in serum because the serum and citrate pools were not obtained from the same subjects. MN concentrations were relatively similar between all techniques and matrices. A single group sent results obtained with a LC-MS method that was presumed to be free of any interferences linked to chromatographic artefacts or immunoassay matrix effects. Results for free MN measured with LC-MS methods were very similar to those observed with HPLC-ECD and immunological methods. Free NMN concentrations were significantly lower with immunological methods, even after correction of spiked results for the D-isomer. The overall recoveries on samples spiked with metanephrines were similar for free NMN and MN, and between the two matrices used for the study ($p = 0.58$ for NMN and $p = 0.14$ for MN). These results indicate that serum may be substituted for citrate plasma. HPLC-ECD methods allowed better recovery compared with immunological method, while LC-MS method provided the highest recovery of spiked free metanephrines (Table 2).

The dispersion of concentrations of total NMN and MN observed between the five laboratories was higher than those obtained with free metanephrines (33%–44% vs. 10%–34%) Table 1.

Plasma free metanephrines represent one of the best biochemical markers for biochemical assessment of a pheochromocytoma. Validation for this assay is difficult because the circulating concentrations of free metanephrines are < 1 nmol/L.

Therefore, it is crucial to implement procedures that will help different laboratories measuring free metanephrines in plasma in order to control the different analytical steps of their assay. Plasma total metanephrines have not been investigated as thoroughly, although they have been described as being potentially sensitive markers for pheochromocytomas (3). External quality control is essential to establish in a ''peer-review'' manner that all results provided by the laboratories involved are relatively similar and accurate. The variability obtained in this preliminary study with plasma free metanephrines was double the variability usually found by PROBIOQUAL for urinary metanephrines, although with a higher number of participants (11). Improvement of the CVs would be necessary to allow better comparisons of the concentrations between the centres.

It appears that free NMN, but not free MN concentrations, were systematically lower with immunoassay independent of the matrix. This fact should be taken into account when patients are followed by different centres. Correction of recovery based on the addition of the racemate of metanephrines into samples should be interpreted with caution, since matrix effects were not taken into account during this calculation. The overall recoveries were similar for

Table 1 Report of the survey on plasma free and total metanephrines.

Laboratory no.	Methods	Free metanephrines, pmol/L								Total metanephrines, pmol/L			
		Sample 1		Sample 2		Sample 3		Sample 4		Sample 1		Sample 2	
		NMN	MN	NMN	MN	NMN	MN	NMN	MN	NMN	MN	NMN	MN
1	RIA	292	139	896	399	240	147	828	503				
2	HPLC ECD	485	180	1540	605	380	165	1455	550				
3	HPLC ECD	600	4097*	1048	7174*	235	1617*	1103	8061*				
4	HPLC ECD	520	117	1327	463	400	213	1143	631				
5	HPLC ECD	720	160	1780	500	560	240	1600	600				
6	HPLC ECD	430	100	1070	390	340	150	970	490	6510	4130	7860	4970
7	HPLC-LC/MS	580	150	1520	550	560	190	1430	600	13,470	5510	14,480	6010
8	HPLC ECD									11,844	1926	10,916	2990
9	HPLC ECD									10,200	5600	9725	3950
10	HPLC ECD									4828	2916	4644	2302
All results	Mean	518	141	1312	485	388	184	1218	562	8346	3643	8286	3553
	SD	136	29	320	85	134	37	283	57	3236	1586	2735	1162
	CV%	26	21	24	17	34	20	23	10	39	44	33	33
Immunological methods	Mean	292	139	896	399	240	147	828	503				
HPLC methods	Mean	551	139	1353	490	383	192	1254	568				
	SD	113	37	313	90	118	42	263	62				
	CV%	20	27	23	18	31	22	21	11				
LC-MS method		580	150	1520	550	560	190	1430	600	13,470	5510	14,480	6010

The results are expressed in pmol/L. Samples 1 and 2 are sera and samples 3 and 4 are plasma citrate. Samples 2 and 4 are spiked with 956 pmol/L of normetanephrine and 456 pmol/L of metanephrine. Plasma free metanephrine obtained from laboratory #3 were excluded since they were very high (HPLC interference). NMN, normetanephrine; MN, metanephrine; RIA, radioimmunoassay; ECD, electrochemical detection; LC-MS, liquid chromatography-tandem mass. *Plasma free metanephrines obtained from laboratory #3 were excluded since they were very high (HPLC interference).

Table 2 Recoveries of plasma free metanephrines expressed in percent of added metanephrines in sera (sample 2) and plasma (sample 4) spiked with 956 pmol/L of normetanephrine and 456 pmol/L of metanephrine.

Laboratory no.	Methods	Samples			
		2		4	
		NMN	MN	NMN	MN
1	RIA	63	57	62	78
2	HPLC ECD	110	93	112	84
3	HPLC ECD	47		91	
4	HPLC ECD	84	76	78	92
5	HPLC ECD	111	75	109	79
6	HPLC ECD	67	64	66	75
7	HPLC-LC/MS	98	88	91	90
All results	Mean	83	76	87	83
	SD	25	14	20	7
	CV%	30	18	22	8
Immunological method		63	57	62	78
HPLC methods	Mean	84	77	91	83
	SD	28	12	20	7
	CV%	33	16	22	6
LC-MS method		98	88	91	90

NMN, normetanephrine; MN, metanephrine; RIA, radioimmunoassay; ECD, electrochemical detection; LC-MS, liquid chromatography-tandem mass.

serum and citrate plasma, but were in favour of HPLC methods compared to immunoassays. This indicates that the lyophilisation process did not cause a significant loss of metanephrines.

Surprisingly, the variability observed with total metanephrines was higher than expected since the concentrations of sulpho-conjugated metanephrines are about 10-fold higher than free metanephrines. These discrepancies may depend on the protocol used for sample preparation. Indeed, differences in efficiency may occur when the procedure used to deconjugate metanephrines consists of perchloric acid hydrolysis followed by heating or treatment with sulphatase.

The variability encountered between assays is most often due to calibrators, independent of the analytical methods used. Therefore, a universal matrix calibrator would definitely help improve the accuracy of MN assays. One of the limitations of our pilot study is the fact that the number of centres involved in the evaluation is relatively low. This makes it difficult to draw definitive conclusions about the variability observed between the groups. However, the Royal College of Pathologists of Australasia (RCPA QAP) has a special program available for plasma free metanephrines in 2009 that may confirm our preliminary results based on a single survey. Another limitation is the fact that the immunological method and the LC-MS method are represented by only one laboratory in this study; these results should be considered with caution.

In conclusion, we have established the conditions necessary to improve harmonisation of the results obtained for plasma free and total metanephrines. Further long-term proficiency tests with more participants should be performed to better evaluate the accuracy observed between laboratories.

Acknowledgements

We are grateful to Dr. Thierry Buclin for statistical assistance. The participants of the study group have equally contributed to this work. The manuscript has been written by EG and co-authors have made appropriate changes.

References

1. Lenders JW, Pacak K, Walther MM, Linehan WM, Mannelli M, Friberg P, et al. Biochemical diagnosis of pheochromocytoma: which test is best? J Am Med Assoc 2002;287:1427–34.
2. Sawka AM, Jaeschke R, Singh RJ, Young WF. A comparison of biochemical tests for pheochromocytoma: measurement of fractionated plasma metanephrines compared to the combination of 24-h urinary metanephrines and catecholamines. J Clin Endocrinol Metab 2003;88:553–8.
3. Peyrin L, Cottet-Emard JM, Pagliari R, Cottet-Emard RM, Badet C, Mornex R. Plasma methoxyamines assay: a practical advance for the diagnosis of pheochromocytoma [in French]. Pathol Biol (Paris) 1994;42:847–54.
4. Lenz T, Zorner J, Kirchmaier C, Pillitteri D, Badenhoop K, Bartel C, et al. Multicenter study on the diagnostic value of a new RIA for the detection of free plasma metanephrines in the work-up for pheochromocytoma. Ann NY Acad Sci 2006;1073:358–73.
5. Manz B, Kuper M, Booltink E, Fischer-Brügge U. Development of enantioselective immunoassays for free plasma metanephrines. Ann NY Acad Sci 2004;1018:582–7.
6. Lenders JW, Eisenhofer G, Armando I, Keiser HR, Goldstein DS, Kopin IJ. Determination of metanephrines in plasma by liquid chromatography with electrochemical detection. Clin Chem 1993;39:97–103.
7. Lagerstedt SA, O'Kane DJ, Singh RJ. Measurement of plasma free metanephrine and normetanephrine by liquid chromatography-tandem mass spectrometry for

diagnosis of pheochromocytoma. Clin Chem 2004;50: 603–11.
8. 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.
9. Grouzmann E, Fathi M, Gillet M, De Torrente A, Cavadas C, Brunner HR, et al. Disappearance rate of catecholamines, total metanephrines, and neuropeptide Y from the plasma of patients after resection of a pheochromocytoma. Clin Chem 2001;47:1075–82.
10. Hoizey G, Lukas-Croisier C, Frances C, Grulet H, Delemer B, Millart H, et al. Study of diurnal fluctuations of plasma methoxyamines in healthy volunteers. Clin Endocrinol 2002;56:119–22.
11. Grouzmann E, Mathian B, Buclin T. Calibration of fractionated metanephrines in urine: still an issue? Clin Chem 2008;54:1745.