CLINICAL STUDY

Phasing-in plasma metanephrines determination

Julie Brossaud^{1,2}, Philippe Gosse³, Blandine Gatta⁴, Antoine Tabarin⁴, Guy Simonnet¹ and Jean-Benoît Corcuff^{1,2}

¹Department of Nuclear Medicine, CHU de Bordeaux, F-33604 Pessac, France, ²Nutrition et Neurobiologie Intégrée, UMR 1286, Université de Bordeaux, F-33000 Bordeaux, France, ³Department of Cardiology/Hypertension, CHU de Bordeaux, F-33000 Bordeaux, France and ⁴Department of Endocrinology, CHU de Bordeaux, F-33604 Pessac, France

(Correspondence should be addressed to J-B Corcuff who is now at Service de Médecine Nucléaire, Hôpital Haut-Lévêque, 33604 Pessac, France; Email: jean-benoit.corcuff@chu-bordeaux.fr)

Abstract

Objectives: We set up plasma normetanephrine (pNMA) and metanephrine (pMA) assays as they demonstrated their usefulness for diagnosing pheochromocytomas. Our scope is to describe some practical laboratory aspects and the clinical relevance of these assays in our endocrinological or cardiological departments.

Methods: We retrospectively reviewed the results of MA from a population of in- and outpatients over a 7-year period. Subjects ($n=2536$) from endocrinological or cardiological departments were investigated (66 pheochromocytomas). Urinary NMA (uNMA) and pNMA, and urinary MA (uMA) and pMA were assayed by HPLC with electrochemical detection.

Results: pNMA and pMA assays are now more frequently requested than uNMA and uMA. This changed our laboratory work load with improved delivery, sensitivity and reliability of plasma assays as well as reduced apparatus maintenance time.

The pNMA and pMA upper reference limits (URLs) of subjects with no pheochromocytoma were 1040 and 430 pmol/l respectively. Sensitivity and specificity based on receiver operating characteristic curves optimal points were 83 and 93% for pNMA at 972 pmol/l and 67 and 98% for pMA at 638 pmol/l. Sensitivity and specificity of paired tests of pMA (positive test: at least one analyte above its URLs) were 100 and 91% respectively.

Conclusion: The very low concentration of analytes requires a sustained very good apparatus analytical sensitivity. This can be obtained in an up-to-date laboratory. In terms of clinical performances, assays in plasma or urine are equivalent. Depending on local preferences, populations, strategies or departments, requests for one or the other assay may sustain the need for specifically defined reference ranges.

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Introduction

Typical pheochromocytoma-induced hypertensive crisis is rare but evocative (1, 2). Alternatively, pheochromocytomas also exhibit less demonstrative cardiovascular features such as chronic hypertension (3, 4). Thus, from a cardiologist or a hypertension specialist point of view, pheochromocytoma should be excluded when certain clinical features are encountered. From an endocrinologist point of view, pheochromocytoma is a possible diagnosis in the presence of an adrenal tumour (5, 6). Alternatively, it is also something to watch for during the lifelong follow-up of subjects with endocrine disorders involving a genetic burden, such as *RET* or *VHL* mutations (7). Subsequently, both the cardiologist and the endocrinologist expect from the biologist the most convenient and the best available biomarker(s) for their purposes.

About 12 years ago, our laboratory established local reference ranges for urinary analytes used for the

diagnosis of pheochromocytoma of patients investigated in cardiology and endocrinology departments (8). This first study was used i) to phase out obsolete assays such as photometric methods or systematic vanilmandelic assay and ii) to re-organise HPLC-ED assays to obtain fast production of results of urinary normetanephrine (uNMA) and urinary metanephrine (uMA) assays.

In 2003, it was suggested that it might be useful to routinely assess NMA and MA in plasma samples (pNMA and pMA) (9). Since then, assays of pNMA and pMA have indeed clearly demonstrated a great potential for the diagnosis of pheochromocytomas and paragangliomas (10, 11, 12, 13, 14, 15, 16, 17, 18). We present the set-up of these assays in 2005, their analytical performances and the main consequences of their routine use in our laboratory. We also present the clinical relevance of these assays to diagnose pheochromocytomas in the endocrinology or cardiology departments of our university hospital.

Subjects and methods

Subjects

We retrospectively reviewed the results of determination of MA from a population of in- and outpatients within a 7-year period (2005–2011) both in the endocrinology and in the cardiology departments. Patients from other departments were not included. The population in which the diagnosis of phaeochromocytoma was questioned resembled the one previously reported (8). It included patients referred for high blood pressure, which did not respond to the usual treatments (chronic as well as acute hypertension) including subjects in the emergency department of cardiology, non-specific signs of phaeochromocytoma (spells, sweating, palpitations, etc.), adrenal tumours and systematic screening for phaeochromocytoma in multiple endocrine neoplasia 2 suffering subjects. Although we could not undertake a detailed investigation, globally, compared with the period investigated in the former study, there were fewer inpatients. Thus, multiple urine collection was no more the rule. As far as possible, anti-hypertensive treatments were discontinued except calcium channel blockers, α -blockers (prazosin) or centrally acting drugs, although no complete retrospective data were available for all patients. Adrenal imaging by computed tomography was also more common (concerning 100% and about 80% of patients in the cardiology and endocrinology departments respectively).

The population of investigated subjects is reported in Table 1. The weight of the 66 phaeochromocytomas was 32 (3–1600) g (median (min–max)); 45% were on the left adrenal, 45% on the right and 10% were bilateral. Fifty per cent of the tumours had a PASS score recorded in their pathological reports (50%, ≤ 3 and 50%, > 3); cancer with no PASS calculated was noted in one. Five patients had genetically confirmed Von Hippel–Lindau diseases, two had MEN2 and one had a *SDHB* mutation; however, for the earlier half of the tumours, no genetic testing could be found. All phaeochromocytomas but one were pathologically confirmed. The non-operated subject had a MEN2 diagnosis with a *RET* mutation, sub-centimetric bilateral adrenal tumours without clinical symptoms

but did not want to be operated yet. Post-operative data of patients with phaeochromocytoma were excluded. To our knowledge, no patient of this cohort was diagnosed with a phaeochromocytoma by other investigators.

Besides the 66 phaeochromocytomas, four neuroblastomas and nine paragangliomas were diagnosed. Among the latter, only two were localised in the abdomen, the others were in the thoracic or cervical regions. Neuroblastomas and paragangliomas were excluded from this analysis because of the small number of patients. Five patients undergoing renal dialysis were also excluded from the analysis.

Analytical methods

Analytes in urines were preserved by adding 30 ml HCl/2 l collection bottle before urine collection. pH was systematically checked before analysis and urine was discarded if pH was > 4.5 . Urine samples were frozen at $-20\text{ }^{\circ}\text{C}$ until assay. Completeness of urine collections was performed in the departments by ensuring that urinary creatinine level was within the reference ranges. For blood sampling, all patients from the cardiology department were sampled 30 min after setting-up an i.v. catheter and while resting in a supine position. In the endocrinological department, the position was more variable: a seated position after a 15-min rest was also adopted for outpatients. Blood was sampled in heparin-containing tubes and immediately stored at $4\text{ }^{\circ}\text{C}$ before centrifugation. Frozen plasma was then stored at $-20\text{ }^{\circ}\text{C}$ until assay.

Total uNMA and uMA were extracted on cationic and anionic columns (Bio-Rad) (8). pNMA and pMA assays were based on a published method (19). Briefly, free pNMA and pMA were extracted on cationic columns from plasma samples after perchloric acid deproteinisation. The eluate was dried out and resuspended in mobile phase. NMA and MA were assayed by reverse-phase HPLC and electrochemical detection (pH 3.8; phase sodium acetate + methanol (10.0–12.5%) using octane sulfonic acid as a counter ion; electrode Bas Biochrom, reference potential 0.80 mV; internal standard: 3 methoxy-4 hydroxy-benzylamine). Inter-assay coefficients of variation (CV) were as follows: for pNMA,

Table 1 Main clinical data of the investigated subjects.

	Subjects with no phaeochromocytoma		Subjects with phaeochromocytomas		Subjects with paragangliomas	Subjects with neuroblastomas
	Cardiology	Endocrinology	Cardiology	Endocrinology	Endocrinology	Endocrinology
No. of subjects	1375	1058	16	50	9	4
No. of samples uNMA	1297	1235	31	132	28	21
No. of samples uMA	1312	1245	31	132	28	21
No. of samples pNMA	571	1672	8	124	36	3
No. of samples pMA	513	1518	7	110	34	2
Sex ratio (F:M)	1.0	1.7	2.0	1.3	0.3	1.0
Age (years)	53	53	51	54	59	18
Median (min; max)	(15; 87)	(15; 89)	(25; 79)	(15; 83)	(22; 73)	(17; 50)

6.9 and 5.9% at 537 and 4669 pmol/l; for pMA, 9.2 and 5.7% at 509 and 4457 pmol/l; for uNMA, 3.6 and 5.7% at 1419 and 6357 pmol/l; for uMA, 5.3 and 5.7% at 433 and 2390 pmol/l. Intra-assay CV were 2.6 and 3.9% at 549 and 4701 pmol/l for pNMA, 4.4 and 6.7% at 486 and 4320 pmol/l for pMA, 1.1% at 1075 pmol/l for uNMA and 1.3% at 879 pmol/l for uMA.

The low limits of quantification for pNMA and pMA were set at 300 pmol/l as it was established when we set up the assay. Since 2010, it has been lower than 300 pmol/l because of improvements of HPLC and electrode, but for the calculations of this study, we kept this less favourable limit.

Statistical analysis and calculations

Calculations and statistics were done using Excel 98, MedCalc (Ostend, Belgium) and Statistica Softwares (Maison-Alfort, France). Non-normally distributed data were log-transformed before using parametric comparison tests. Significant differences were validated for $P < 0.05$.

All samples were taken into account individually including those from patients that had multiple samplings. No systematic number of samples per patient was decided when a pMA assay was set up. Thus, some patients had several sampling and all results were used for the calculations. For calculations, we choose to use the method that seemed the closest to the method used by all the clinicians in the departments; i.e. if any sample was above the reference values, it could be considered suspect. Alternative methods (to use a random sample, the first sample and the mean of samples) mirrored less closely the clinical approach used in our departments. The upper reference limits (URLs) were calculated according to the double-sided non-parametric method (Clinical and Laboratory Standards Institute guideline C28-A3).

Receiver operating characteristic (ROC) curves were established for individual analytes using MedCalc. Diagnostic sensitivity and specificity were also estimated for paired measurements of metabolites (NMA and MA) either in plasma or in urine. Positive tests were defined by at least one of the two metabolites in the pair being above the chosen cut-off; negative tests were defined by both metabolites being below the chosen cut-off.

ROC curve comparisons were performed for patients who supplied both urine and plasma samples within a 48-h period (with pheochromocytoma, 82 samples; without pheochromocytoma, 677 samples).

Results

Laboratory considerations

A total of 2536 patients (1403 F) were investigated from the cardiology and endocrinology departments,

thus generating 3059 uNMA and uMA, and 2817 pNMA and pMA assays.

Plasma assays of NMA and MA were set up in 2005 and are now more frequently requested than their urinary counterpart (Fig. 1). This had technical as well as clinical consequences. First, we had to change (in 2010) the distribution of workload during the week. Initially, the series of assays were more frequent for urine than for plasma. When this proportion was inverted, we had to less frequently change the mobile phase as well as the potential of the electrodes. This reduced the electrochemical noise and improved the analytical sensitivity (especially useful for pMA). This also allowed us to less frequently change the HPLC columns, thus reducing the cost of the analysis. Secondly, the increased number of plasma assays was at least in part due to an increase in the number of outpatients vs inpatients. Our change of workload resulted in faster results for most outpatients and plasma results more synchronous with other data obtained during the single day of hospitalisation (Fig. 1). The median delay between sampling and results is now < 1 week. The longest delays are mainly due to pre-analytical transport, bank holidays or re-analysis with dilutions because of elevated analyte levels. Because of possible back-up, apparatus failure exceptionally causes delays longer than 2 days.

Clinical considerations

The URLs (95th CI) of the analytes, NMA or MA either in urine or in plasma from subjects with no tumour were calculated. The uNMA and uMA URLs were 3179

Figure 1 Chronological evolution of the number of samples and of the delay between sampling and delivery of results. Number of samples per year (left ordinate axis): closed squares, urinary samples and open squares, plasma samples. Delay (d, right ordinate axis): median delay dotted line, triangles and 5th and 95th percentiles of the delay delimiting the grey area. Note the increased delay in 2010 that led to the reorganisation of the series of assays.

and 1096 nmol/day. The pNMA and pMA URLs were 1040 and 430 pmol/l.

Because not all patients had all urinary and plasma analytes assayed within a short period, the performances of the tests were first investigated for each analyte taking into account all available data for patients with or without a phaeochromocytoma (Fig. 2 and Table 2). Area under the ROC curves (ROC AUC) for concentrations of NMA, MA and main associated sensitivities and specificities are presented in Fig. 3 and Table 3. Calculation of diagnostic sensitivity and specificity based on paired analysis was also performed; for at least one analyte above its URLs, a given pair pNMA–pMA was considered positive. The respective sensitivities and specificities were then: 94.5 and 90.9% and 100 and 90.9% for MA in urine and plasma respectively.

Statistical comparison of ROC curves was performed for patients who had both urine and plasma samples taken within a 48-h period: pNMA, uNMA performed similarly, pMA and uMA performed identically and more poorly. AUC based on test pairs were similar to pNMA and uNMA AUC.

In most cases, the clinical reasons supporting an investigation in cardiology or endocrinology departments are different. Then, the type of biological investigation is often different. We, however, wanted to assess a crude picture of the status of the metabolites in

the two departments. We compared NMA or MA levels between subjects without phaeochromocytoma from cardiological or endocrinological wards. All analytes were similar between groups except $P < 0.05$ for uMA. ROC curves and the various thresholds expressed in Table 3 were also very similar when the diagnosis of the whole population of phaeochromocytoma was opposed to the patients without phaeochromocytoma either from the cardiology or endocrinology departments. We did not perform an analysis of the sensitivity and specificity curves with phaeochromocytomas for each department because of the relatively low number of plasma samples collected at the same time as urine samples in the cardiological department. However, it is interesting to note that many analytes were different in urine or plasma from subjects with phaeochromocytomas between the two departments (Table 2), probably reflecting the different recruitments of patients.

Discussion

This study describes the performances of NMA and MA assays in plasma or urine to diagnose phaeochromocytomas in two departments of a university hospital. It confirms, if needed, that urine assays are sound methods for this purpose (8, 10, 11, 12, 13, 15, 17, 18, 20). This study also confirms a clear usefulness of plasma assays in a major clinical centre as already demonstrated (10, 11, 12, 13, 15, 17, 18). How can such a retrospective study help centres not yet routinely assaying pMA?

Laboratory considerations

In our laboratory, plasma assays outnumber urine assays. In our experience, the main reason for this substitution is its convenience in 1-day hospitalisation.

How do our results about pNMA and pMA assays compare with other published studies? The ROC AUC of our data are in general agreement with others' publications both in terms of AUC and ranked performance of the analytes (11, 15, 21, 22). On the other hand, the sensitivities and specificities linked to given concentrations may differ in the various publications including this one. Different publications use the URLs as thresholds. In our case, the optimal thresholds and the URLs are a bit different, which results in slightly different couples of sensitivities and specificities. This is probably due to the populations investigated including the population referred and proportions of sporadic and hereditary phaeochromocytomas. Thus, it must be kept in mind that the concentration thresholds and attached sensitivities and specificities established in one given laboratory should be considered as local indicators rather than universal values. Furthermore, in the past, difficulties to subscribe to external quality programmes for pMA and lack of certified reference material made it

Figure 2 Concentrations of normetanephrine (NMA) and metanephrine (MA) of all subjects either in urine or in plasma (upper or middle panels). Subjects with phaeochromocytoma from the cardiological department (open circles), the endocrinological department (closed circles) or no phaeochromocytoma (dots).

Table 2 Biological data of the investigated subjects presented as mean (minimum; maximum). Paragangliomas and neuroblastomas were investigated in the endocrinological department.

	Subjects with no pheochromocytoma		Subjects with pheochromocytomas		Subjects with paragangliomas	Subjects with neuroblastomas
	Cardiology	Endocrinology	Cardiology	Endocrinology	Endocrinology	Endocrinology
uNMA	1322 (68; 14 971)	1365 (39; 6123)	12 650 ^b (3916; 338 310)	6518 ^b (916; 88 336)	5481 (1594; 8774)	6282 (1029; 341 656)
uMA	525 ^a (25; 4298)	487 ^a (17; 1724)	3158 (316; 359 047)	3000 (34; 61 969)	662 (328; 1108)	642 (331; 4823)
pNMA	435 (300; 2518)	447 (300; 4720)	7309 (1391; 50 529)	3512 (410; 80 993)	1817 (456; 4035)	881 (628; 1936)
pMA	300 (300; 1117)	300 (300; 2198)	2901 ^b (300; 20 088)	1155 ^b (300; 13 362)	300 (300; 534)	851 (300; 1402)

NMA, normetanephrine; MA, metanephrine; n, number of available data. $P < 0.05$ cardiological vs endocrinological departments: ^asubjects without pheochromocytoma, ^bsubjects with pheochromocytoma.

difficult to perform direct comparisons of concentrations between publications (23). This has now changed with the availability of an external quality programme by the Royal College of Pathologists of Australasia Quality Assurance Program (24).

Clinical considerations

How should we improve our routine clinical practice? First, no clear advice about the patients' posture during sampling was given to the departments when we set up this assay. It has been reported that a seated posture may increase the false-positive rate of pMA (16). In the cardiological departments, all patients were sampled 30 min after set-up of an i.v. catheter and rest in a supine position. In the endocrinology department, the position was more variable, and the seated position was also used for outpatients. As other assays, such as renin and aldosterone, are likely to be performed at some point when investigating hypertension or adrenal tumours, it may be easy to obtain a rested supine posture for all patients. An interesting point is that diet does not affect free pNMA and pMA (25), which is particularly suitable for outpatients.

Secondly, should a clinical decision be made using a simultaneous analysis of both NMA and MA and their position with regard to their respective URL (26, 27, 28)? The improvement upon considering individual analytes is indeed especially noticeable on the specificity attached to 100% sensitivity. We used to routinely propose a graphical approach for urine total MA (NMA and MA axis) (8). A very similar graph can be drawn for plasma-free MA (16). This approach with urine total MA, useful to less experienced practitioners, is then likely to be proposed again for plasma-free MA.

Thirdly, what should be done for the routine follow-up of individuals and families with hereditary paragangliomas and pheochromocytoma? Indeed, it has been shown that depending on the genetic abnormality, the tumours preferentially secrete NMA or MA (14, 17, 29). To our knowledge, there is no special survey of the modification of pNMA or pMA levels during the follow-up of subjects with mutations although but still not presenting a tumour.

Figure 3 ROC curves and their areas of urinary and plasma normetanephrine (NMA) and metanephrine (MA) concentrations the diagnosis of pheochromocytoma. Upper panel, individual analytes and lower panel, paired analytes according to the logistic regression analysis.

Table 3 Biological thresholds and associated sensitivities and specificities for the various parameters. Urine threshold units (nmol/day) and plasma threshold units (pmol/l).

	<i>n</i>		AUC		100% sensitivity		Optimal point			100% specificity	
	No phaeo-chromocytoma	Phaeochromocytoma	Value	95% CI	Level	Specificity (%)	Level	Specificity (%)	Sensitivity (%)	Level	Sensitivity (%)
uNMA	3722	163	0.944	0.937–0.952	915	26	2950	93	83	15 567	25
uMA	3751	163	0.812	0.799–0.824	33	0	1798	99	57	4298	38
pNMA	2607	132	0.940	0.930–0.949	410	44	972	93	83	4720	37
pMA	2249	117	0.843	0.828–0.858	300	0	638	98	67	1507	43

NMA, normetanephrine; MA, metanephrine; AUC, area under the curve.

Thus, considering the relatively low number of patients seen in each centre, the best approach could possibly be to establish long-term within-subject variances of analytes to alert when the levels are statistically and repeatedly increased.

Lastly, should phasing-in plasma assays be accompanied by phasing-out urine assays? Some papers have reported a superiority of plasma-free MA assay compared with urine total MA assay (21, 30). This study also follows this trend. However, this significant but modest improved performance is not the only question to be considered on a practical point of view: other considerations should be taken into account as stated during an international symposium on phaeochromocytoma (16). It is thus unlikely that this point will be universally answered. Indeed, although performing much better than plasma catecholamines, pMA are not completely independent of physical (sampling position) and probably psychological stress (9). Such a minor and episodic stress detected by plasma assays may be of less importance and smoothed in a day-long urine collection. Considering the various populations investigated, the proclivities of practitioners and the technical difficulties of plasma vs urine assays, both using the same analytical apparatus, phasing-in plasma assays will not yet if ever phase out urine assays in our laboratory.

Clinical Considerations

Obviously, this study suffers from some limitations, most of them related to its retrospective nature. First, there were some evolving disparities between the diagnostic strategies of two departments about clinical, biological and imaging investigations. For instance, earlier patients did not have the benefit of systematic imaging. Although no tumour was discovered during follow-up, small, non-significantly secreting or extra abdominal tumours may have been overlooked; a possible minor decrease in test performances is thus possible. Secondly, analytical procedures also improved especially for plasma assays as, for instance, we acquired more sensitive analytical devices. Thus, the lower reference range and median pNMA and pMA values of subjects without phaeochromocytoma are probably lower than what is related here. However, this does not affect the

upper reference range and related diagnosis probability. The results of urine MA were also consistent with the local reference range previously established (8). Thirdly, perceived stress was likely responsible for elevated levels of MA in the cardiological ward. It is possible that such a perceived stress could increase the number of subjects with high levels of MA in the cardiological ward. Although no sampling is usually done on arrival, no reliable data could be retrospectively obtained about this point. Possibly even more importantly, physiological distress in cardiology wards (inadequate cardiac output, cardiac ischaemia or arrhythmias) associates with an activated sympathetic nervous system and may contribute to elevated levels of MA.

In conclusion, to set up an assay of NMA and MA in plasma is not out of reach of a laboratory used to perform this assay in urine. However, the very low concentration of analytes requires maintaining a day-to-day very good apparatus analytical sensitivity through skilled technicians. Economic implications of alternate screening strategies for phaeochromocytoma evaluated algorithms and costs in hypertensive patients. The main conclusion is the necessity to focus on high-risk patients. Obviously, to evaluate costs to diagnose phaeochromocytomas among incidentalomas would elicit different costs as by definition adrenal imaging is already performed. From a clinical point of view, no clear evidence favours an all-plasma or an all-urine strategy to diagnose a phaeochromocytoma: both assays will coexist in most laboratories. Depending on local preferences, populations, strategies, departments, etc., requests for one or the other assay may occur. Thus, in this retrospective evaluation, we confirm that to assay MA in plasma is at least as useful as to assay them in urine. Clinical departments should support their laboratories to perform them. Would it be useful to establish different thresholds, rules and strategies for patients with hypertension or incidentaloma or hereditary risk of phaeochromocytoma? Indeed, it seems that, at least in our hospital, the departments dealing with endocrine or hypertension do not recruit phaeochromocytomas with the same level of NMA and MA secretion. Whether applying different rules to different departments improves phaeochromocytoma diagnosis thus remains to be established.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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