

RESEARCH ARTICLE

Optimising urinary catecholamine metabolite diagnostics for neuroblastoma

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Funding information

Villa Joep Foundation

Abstract

Introduction: The analysis of urinary catecholamine metabolites is a cornerstone of neuroblastoma diagnostics. Currently, there is no consensus regarding the sampling method, and variable combinations of catecholamine metabolites are being used. We investigated if spot urine samples can be reliably used for analysis of a panel of catecholamine metabolites for the diagnosis of neuroblastoma.

Methods: Twenty-four-hour urine or spot urine samples were collected from patients with and without neuroblastoma at diagnosis. Homovanillic acid (HVA), vanillylmandelic acid (VMA), dopamine, 3-methoxytyramine, norepinephrine, normetanephrine, epinephrine and metanephrine were measured by high-performance liquid chromatography coupled with fluorescence detection (HPLC-FD) and/or ultra-performance liquid chromatography coupled with electrospray tandem mass spectrometry (UPLC-MS/MS).

Results: Catecholamine metabolite levels were measured in urine samples of 400 neuroblastoma patients (24-hour urine, $n = 234$; spot urine, $n = 166$) and 571 controls (all spot urine). Excretion levels of catecholamine metabolites and the diagnostic sensitivity for each metabolite were similar in 24-hour urine and spot urine samples

Abbreviations: Amsterdam UMC, Amsterdam University Medical Centre; AUC, area under the curve; HPLC-ED, high-performance liquid chromatography coupled with electrochemical detection; HPLC-FD, high-performance liquid chromatography coupled with fluorescence detection; HVA, homovanillic acid; ROC, receiver-operating characteristic; SIOPEX, International Society of Paediatric Oncology European Neuroblastoma; UPLC-MS/MS, ultra-performance liquid chromatography coupled with electrospray tandem mass spectrometry; VMA, vanillylmandelic acid.

[#]Collaborators are listed in Supporting Information.

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($p > .08$ and $>.27$ for all metabolites). The area under the receiver-operating-characteristic curve (AUC) of the panel containing all eight catecholamine metabolites was significantly higher compared to that of only HVA and VMA (AUC = 0.952 vs. 0.920, $p = .02$). No differences were observed in metabolite levels between the two analysis methods.

Conclusion: Catecholamine metabolites in spot urine and 24-hour urine resulted in similar diagnostic sensitivities. The Catecholamine Working Group recommends the implementation of spot urine as standard of care. The panel of eight catecholamine metabolites has superior diagnostic accuracy over VMA and HVA.

KEYWORDS

catecholamine metabolites, diagnostic sensitivity, metanephrines, neuroblastoma, tandem mass spectrometry, urine collection

1 | INTRODUCTION

Neuroblastoma is the most common extra-cranial paediatric solid tumour with a median age of 18 months at diagnosis.^{1,2} Measurement of catecholamine metabolites in urine is a cornerstone in the diagnostic workup of neuroblastoma. Elevated levels of urinary catecholamine metabolites are detected in 95% of patients with neuroblastoma at diagnosis.^{3,4}

Historically, the use of catecholamine metabolite measurements in 24-hour urine has been recommended for the diagnostic workup of neuroblastoma, because circadian fluctuations in the excretion of urinary catecholamines have been observed in healthy adult controls.⁴⁻⁶ Frequently, the collection of urine during a 24-hour period has been shown to be challenging.⁷ Due to the young age of patients with neuroblastoma at diagnosis, 24-hour urine collection frequently requires a catheter or the use of several urine collection bags, which increases the chance of failure to complete the 24-hour collection period.⁸⁻¹⁰ Another approach for the analysis of catecholamine metabolites is the use of randomly collected spot urine samples (i.e., spot urines) for analysis of catecholamine metabolites, with metabolite excretion expressed relative to creatinine excretion. Several previous studies showed that the diagnostic sensitivity of the most widely used urinary markers, homovanillic acid (HVA) and vanillylmandelic acid (VMA) is similar between the use of spot urine and 24-hour urine, suggesting that at least for HVA and VMA, the sampling of spot urine may be used instead of 24-hour urine.^{8,9,11-13} However, whether only HVA and VMA should be included in the diagnostic workup of neuroblastoma is currently debated, because their combined diagnostic sensitivity is only 85% (in 24-hour and spot urine).^{3,11,14} By analysing eight urinary catecholamine metabolites in an extended panel, including HVA and VMA but also their precursors dopamine, 3-methoxytyramine, norepinephrine, normetanephrine, epinephrine and metanephrine, the diagnostic sensitivity increased to 95%.^{3,15} Furthermore, we have shown in several cohorts that elevated urinary 3-methoxytyramine at diagnosis correlated with poor prognosis, even in patients with high-risk neuroblastoma.^{16,17} The specificity of the extended panel has not been described before and it is not yet known if spot urine measurements of the extended panel can be reliably used for neu-

roblastoma diagnostics. Due to differences in sample collection time, measurement methods and catecholamine metabolite panels, the analysis of catecholamine metabolites may differ between neuroblastoma treatment centres. There are several methods for quantitative analysis of urinary catecholamine metabolites, these methods include high-performance liquid chromatography coupled with fluorescence detection or electrochemical detection (HPLC-FD/ED) and ultra-performance liquid chromatography coupled with electrospray tandem mass spectrometry (UPLC-MS/MS). HPLC-FD is widely used but this technique is very time-consuming and several common drugs may cause interference with the detection of catecholamine metabolites.¹⁸⁻²⁰ The detection, accuracy and precision of urinary biogenic amines have improved with implementation of mass spectrometry.²¹

The current study has three aims, namely (i) to analyse the diagnostic sensitivity of the panel of eight catecholamine metabolites between spot urine and 24-hour urine, (ii) to evaluate the diagnostic accuracy in spot urine of the panel of eight catecholamine metabolites in comparison with the classic panel of HVA and VMA, and (iii) to establish an overview of sample collection methods, detection methods and catecholamine metabolite panels in centres associated with the International Society of Paediatric Oncology European Neuroblastoma (SIOPEN). Here, we address these aims in a first consensus paper from the SIOPEN Catecholamine Working Group.

2 | METHODS

2.1 | Subjects

We retrospectively studied 400 patients with neuroblastoma who were diagnosed and treated between 1990 and 2014 at the Amsterdam University Medical Centre (Amsterdam UMC), and between 2018 and 2021 at the Princess Maxima Centre. All patients were diagnosed according to the international neuroblastoma staging system (INSS) with histologically confirmed neuroblastoma. The Amsterdam UMC cohort included 296 patients with neuroblastoma and was described earlier.^{3,15} The Princess Maxima Centre cohort included 104 patients

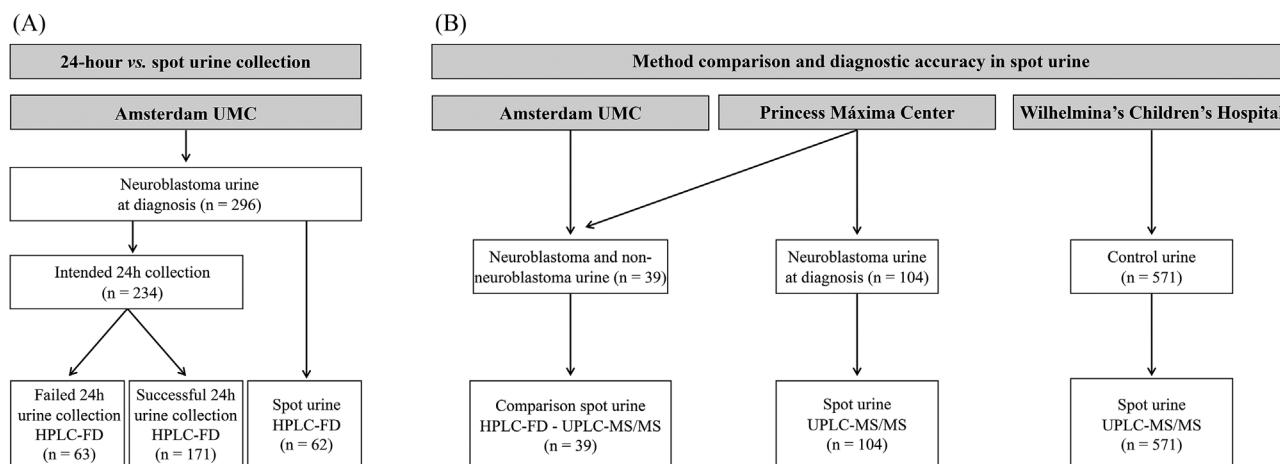


FIGURE 1 Urine samples flowchart. (A) Urine samples from neuroblastoma patients were collected at diagnosis as intended 24-hour urine collection ($n = 234$) or spot urine collection ($n = 62$) and analysed by HPLC-FD (high-performance liquid chromatography coupled with fluorescence detection). (B) Neuroblastoma and non-neuroblastoma (i.e., patients with other paediatric cancer types or no malignancy) spot urine samples ($n = 39$) were used for method comparison. Spot urine samples from neuroblastoma patients were collected at diagnosis in the Princess Maxima Centre ($n = 104$), and spot urine samples from controls ($n = 571$) were collected in the Wilhelmina's Children's Hospital and analysed by UPLC-MS/MS (ultra-performance liquid chromatography coupled with electrospray tandem mass spectrometry).

with neuroblastoma. In addition, a separate cohort of 39 neuroblastoma and non-neuroblastoma patients (i.e., patients with other paediatric cancer types or no malignancy) from the Amsterdam UMC and Princess Maxima centre was used for methodological comparison of HPLC-FD and UPLC-MS/MS (see Supporting Methods, Table S1). Finally, a group of 571 patients from the Wilhelmina Children's Hospital without neuroblastoma who were screened for metabolic disease was used as a control group to establish age-dependent reference values for the catecholamine metabolite concentrations (Table S2). An overview of the cohorts is provided in Figure 1. The medical ethics committees of the AUMC (Reference number: W16_093#16.112) and Princess Maxima Centre (Reference number: PMCLAB2019.075) declared that the Medical Research Involving Human Subjects Act does not apply to this study.

2.2 | Urine sample collection and biochemical assays

The 24-hour urine or spot urine (a single, randomly collected specimen) samples were collected at time of neuroblastoma diagnosis. Collection time was standardly reported on the application form. Failed 24-hour urine collection was defined as a collection period of less than 24 hours or longer than 24 hours because additional samples were collected after previous missed ones. No dietary restrictions were required prior to urine collection for all patients. At the Amsterdam UMC, 24-hour urine and spot urine samples were collected and analysed by HPLC-FD. Samples were protected from light, acidified using hydrochloric acid and stored at 4°C until analysis, as previously described.³ At the Princess Maxima Centre and the Wilhelmina Children's Hospital, spot urine samples were collected and analysed by UPLC-MS/MS.²² Sam-

ples were protected from light without prior acidification and stored at -20°C until analysis. In all three laboratories, urine samples were analysed within 1 week after collection. No 24-hour and spot urine samples were available from the same patients.

2.3 | Analysis of data

We performed a retrospective analysis of neuroblastoma patient data that was gathered as part of standard of care. In addition, for methodological comparison of HPLC-FD and UPLC-MS/MS, catecholamine metabolite measurements in banked urine samples from neuroblastoma and non-neuroblastoma patients were analysed ($n = 39$, see Supporting Methods). Catecholamine metabolite concentration was expressed relative to creatinine excretion. A catecholamine metabolite was considered to be elevated when the concentration exceeded the upper limit (95th percentile) of the age-specific reference range for 24-hour urine and for spot urine samples.³ Age-dependent reference values for catecholamine metabolites in 24-hour urine were previously published.³

2.4 | Survey to SIOPEN centres

In order to investigate the methods used for analysis of catecholamine metabolites, a survey was sent to SIOPEN centres. A survey was sent by email to 35 centres associated with the Catecholamine Working Group regarding the methods of urine collection, the methods applied for the detection of catecholamine metabolites and which catecholamine metabolites have been included in the clinical diagnostic test for neuroblastoma.

2.5 | Statistical analysis

The chi-square test was used to compare patient characteristics between groups. The Mann-Whitney *U* test was used to compare catecholamine metabolite excretion levels between groups. Diagnostic sensitivity was defined as the number of patients with elevated metabolite(s) levels divided by the total number of patients with confirmed neuroblastoma. Diagnostic specificity was defined as the number of controls without any elevated metabolite divided by the total number of controls. Confidence intervals of the sensitivity and specificity were calculated using the Wald method. An area under the receiver-operating characteristic (ROC) curve (AUC) was generated to evaluate diagnostic accuracy. The method of DeLong et al. was used to calculate the differences between the AUCs.²³ The chi-square test was used to compare the diagnostic sensitivities between groups. Statistical analyses were performed using GraphPad Prism (GraphPad Software, version 7.03). A *p*-value less than .05 was regarded as statistically significant.

3 | RESULTS

3.1 | Catecholamine metabolite analysis in centres associated with SIOPEN

A response was received from 21 out of 35 centres that were contacted (60%). The survey showed that seven out of 21 centres measured catecholamine metabolite concentrations in 24-hour urine or a combination of spot and 24-hour urine (Figure 2). HPLC-UPLC-MS/MS was the most often used detection method. At least six different panels of catecholamine metabolites were used in SIOPEN centres and the most frequently used were the panel of eight metabolites and the panel of HVA and VMA.

3.2 | Patient characteristics and feasibility of 24-hour urine collection

Patient characteristics are shown in Table S3. Twenty-four-hour urine collection was unsuccessful in 63 of the 234 patients (27%) (Figure 3A). Failure to complete 24-hour urine collection was observed more often in female patients (Figure 3B, *p* = .005) and in patients with stage 4 disease (Figure 3C, *p* = .003), but it was not associated with other clinical characteristics (Table S4). Strikingly, failure to collect 24-hour urine occurred in 20%–40% of attempted collections in all age groups, indicating that this collection method is not only problematic in infants, but also in older patients (Figure 3D).

3.3 | Catecholamine metabolites in spot urine samples versus 24-hour urine collection

To test if the panel of eight catecholamine metabolites could also be reliably analysed in spot urine samples, we compared catecholamines

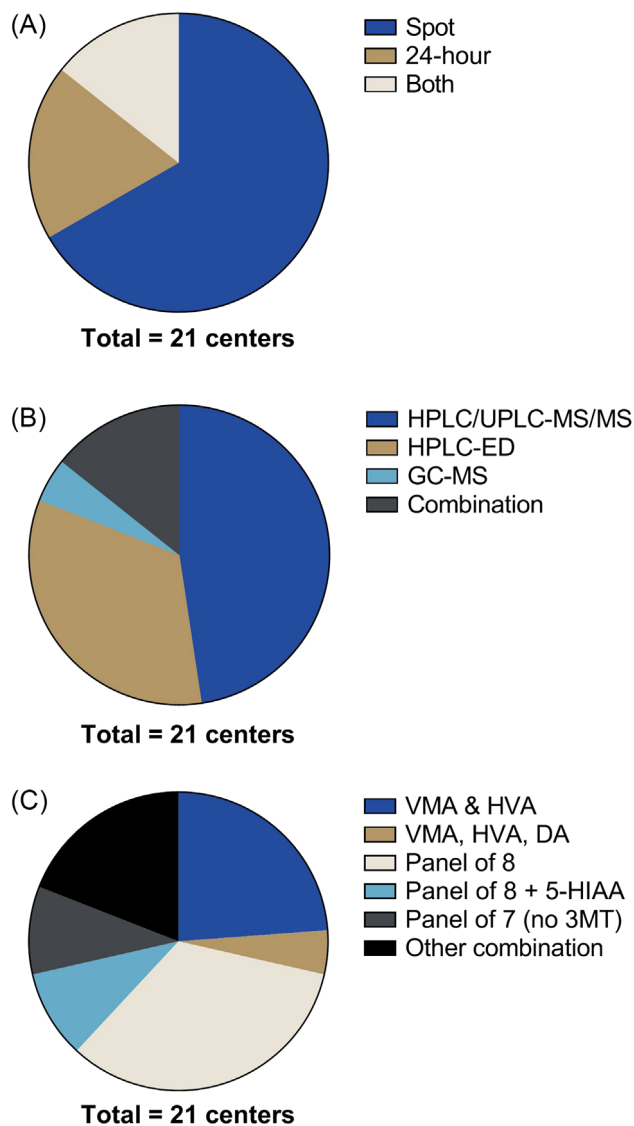


FIGURE 2 Overview of catecholamine analysis in 21 SIOPEN (International Society of Paediatric Oncology Europe Neuroblastoma Group) centres. Twenty-one centres associated with the SIOPEN responded to a survey about catecholamine metabolites. (A) Urine collection method, (B) analysis method, and (C) catecholamine metabolites used for diagnosing neuroblastoma.

concentrations in 24-hour urine (*n* = 171) and spot urine samples (*n* = 62) from the Amsterdam UMC cohort (Figure S1A–H). There were no differences in clinical characteristics between both groups (Table S4). Both the catecholamine excretion values (Figure S1A–H) and the resulting diagnostic sensitivities (Figure 4) for all eight catecholamine metabolites were comparable for spot urine and 24-hour urine samples.

3.4 | Comparison of two diagnostic panels

To confirm that catecholamine metabolite measurements of both analysis methods may be used for comparative analysis, a correlation

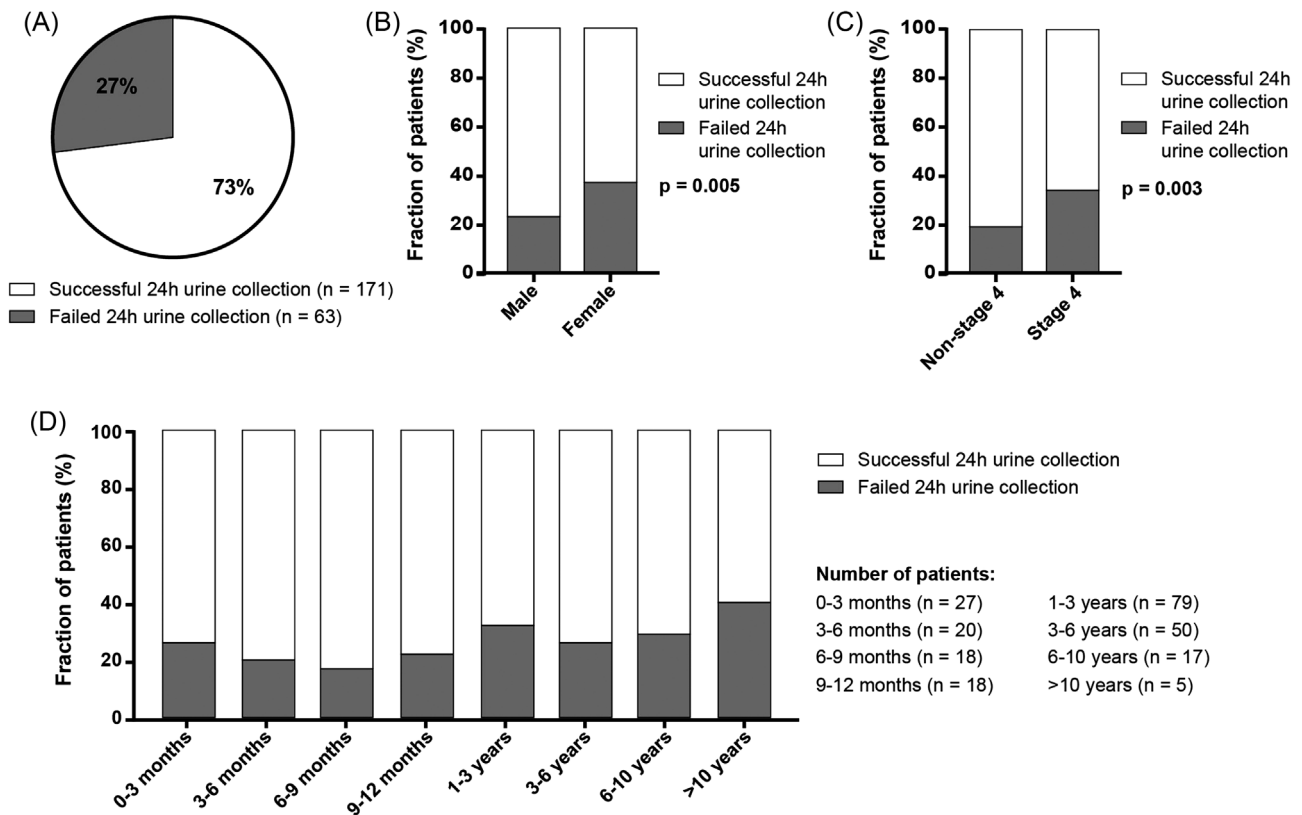


FIGURE 3 Success rate of diagnostic 24-hour urine collection in neuroblastoma patients. (A) At time of diagnosis, 24-hour urine collection was started in 234 neuroblastoma patients. The fraction of patients in whom 24-hour urine collection was completed successfully and failed are indicated in white and grey, respectively. (B) Success rate of 24-hour urine collection when stratified based on patient gender. (C) Success rate of 24-hour urine collection when stratified based on stage of disease. (D) Success rate of 24-hour urine collection in different age groups.

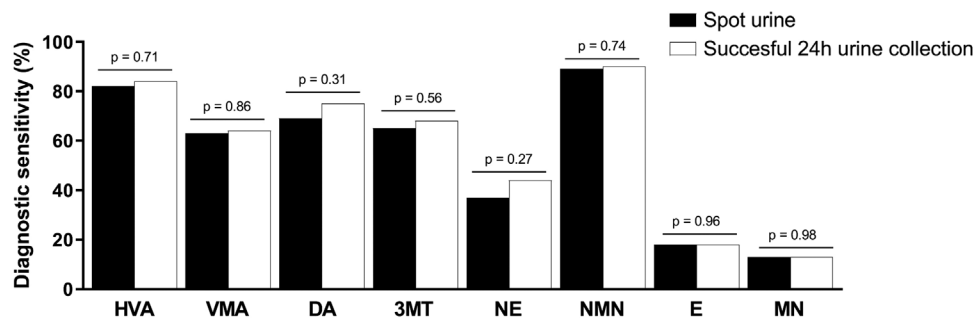


FIGURE 4 Effect of mode of urine collection on diagnostic sensitivity for neuroblastoma. Urine was collected at diagnosis as spot urine ($n = 62$, black) or as successful 24-hour urine collection ($n = 171$, white) and analysed by HPLC-FD (high-performance liquid chromatography coupled with fluorescence detection). Diagnostic sensitivity was defined as number of patients with elevated metabolite(s) divided by total amount of patients. p -Values are given.

analysis was performed in simultaneously analysed samples from a separate cohort (correlation between HPLC-FD and UPLC-MS/MS measurements $p < .001$ and $R \geq .83$ for all eight catecholamine metabolites; Supporting Methods, Figure S2). Catecholamine metabolite measurements by HPLC-FD and UPLC-MS/MS resulted in similar diagnostic sensitivities (Figure S3). The diagnostic sensitivity and specificity of the panel containing only HVA and VMA and the panel containing all eight catecholamine metabolites is shown in Table 1.

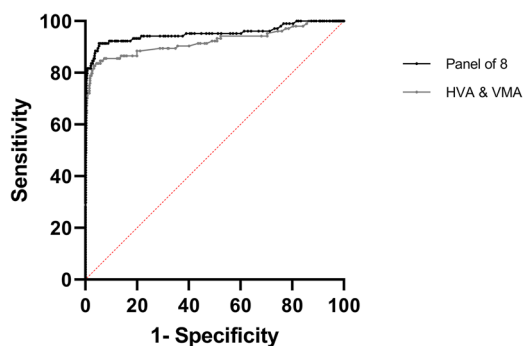
Diagnostic sensitivity was improved by inclusion of six catecholamine metabolites, in addition to VMA and HVA, using either spot urine or 24-hour urine samples. Diagnostic specificity of the panel containing only HVA and VMA was higher than that observed for the panel containing all eight metabolites (Table 1). The diagnostic accuracy of the panel with eight catecholamines was higher than the panel containing only VMA and HVA (AUC 0.952 and 0.920, respectively, $p = .02$; Figure 5). Regardless of the mode of urine collection and

TABLE 1 Diagnostic sensitivity and specificity of two panels of urinary catecholamine metabolites.

	HVA + VMA	Panel of 8 metabolites
24-Hour urine HPLC-FD		
Sensitivity (n = 171)	85 (78.6–89.5)	95 (90.9–97.8)
Spot urine HPLC-FD		
Sensitivity (n = 62)	84 (72.6–91.2)	94 (84.1–97.9)
Spot urine UPLC-MS/MS		
Sensitivity (n = 104)	86 (77.5–91.2)	94 (87.7–97.6)
Specificity (n = 571)	92 (89.4–93.9)	75 (71.4–78.5)

Note: Sensitivity and specificity in percentage with 95% confidence intervals in brackets. The panel of 8 catecholamine metabolites consisted of homovanillic acid, vanillylmandelic acid, dopamine, 3-methoxytyramine, norepinephrine, normetanephrine, epinephrine and metanephrine. Urine samples were considered positive if at least one of the catecholamine metabolites exceeds the upper reference value.

Abbreviations: HPLC-FD, high-performance liquid chromatography coupled with fluorescence detection; HVA, homovanillic acid; UPLC-MS/MS, ultra-performance liquid chromatography coupled with electrospray tandem mass spectrometry; VMA, vanillylmandelic acid.

**FIGURE 5** Comparison of diagnostic performance of two panels of catecholamine metabolites. Receiver-operating curves (ROC) are shown. The panel of eight catecholamine metabolites consists of homovanillic acid (HVA), vanillylmandelic acid (VMA), dopamine, 3-methoxytyramine, norepinephrine, normetanephrine, epinephrine and metanephrine.

analysis method, normetanephrine and HVA were the most commonly elevated metabolites in neuroblastoma (Figure S4A–C). In addition, the specificity per catecholamine metabolite varied between 94% and 96% with the highest specificity for normetanephrine and HVA (Figure S5).

4 | DISCUSSION

In this study, we demonstrated that neuroblastoma diagnostics can be performed faster and in a more patient-friendly way by shortening the duration of urine collection from 24 hours to collection of spot urine. Furthermore, we showed that the diagnostic accuracy of the panel of eight catecholamine metabolites was significantly higher compared to the combined use of only HVA and VMA.

In line with previous studies, we showed that 24-hour urine collection could not be completed in more than 25% of the neuroblastoma patients.^{8,9} Failure to complete the 24-hour urine collection period occurred more often in female patients and patients with metastatic disease (stage 4), which might be attributed to anatomical challenges during urine collection and poor clinical condition at time of diagnosis, respectively.²⁴ Although failure of 24-hour urine collection has been mainly attributed to the young age of patients with neuroblastoma and the necessity to use urine collection devices,¹⁰ this study showed that failure of 24-hour urine collection occurred with a similar prevalence in all age groups. This shows that 24-hour urine collection is difficult to complete in all neuroblastoma patients.

The recommendation to use 24-hour urine collection for neuroblastoma diagnostics was based on the observation that under normal conditions, excretion of some urinary catecholamine metabolites follows a circadian rhythm.^{5,6} However, in neuroblastoma patients, Itoh and Omori showed that neuroblastoma cells have a relatively low storage capacity and suggested that the catecholamine excretion in neuroblastoma is continuous.²⁵ In adult patients with pheochromocytoma and paraganglioma, Peitzsch et al. compared the daytime and nighttime urinary catecholamine excretions from patients with healthy controls and observed that differences between daytime and nighttime urinary excretion for norepinephrine, epinephrine, dopamine, normetanephrine and metanephrine were present in controls.²⁶ In patients with pheochromocytoma and paraganglioma, only a significant difference in day-to-night variation in excretion was observed for norepinephrine and epinephrine.²⁶ In neuroblastoma diagnostics, norepinephrine and epinephrine contribute to a very small extent to the diagnostic sensitivity.³ For the other catecholamine metabolites, such as 3-methoxytyramine, HVA and VMA, no differences in day-to-night variability were reported between patients and controls.^{26,27} Furthermore, Monsaingeon et al. showed that analyses of urinary catecholamine metabolites in consecutive spot urines from patients with neuroblastoma did not show significant fluctuations over the day.¹⁰ These findings are in line with our observation that no differences were observed in the diagnostic sensitivity of spot urine sampling compared to 24-hour urine collection.

Various studies, including this study, showed that the analysis of HVA and VMA in spot urine has equivalent diagnostic sensitivity when compared to 24-hour urine collection.^{8,9,11,12} In addition, our study also demonstrated that the panel of eight urinary catecholamine metabolites can be analysed in spot urine, instead of 24-hour urine collection, without compromising diagnostic sensitivity. Although the feasibility of catecholamine metabolite analysis in spot urine during therapy and follow-up has not been investigated, the continuous excretion of catecholamines in neuroblastoma would most likely result in a comparable sensitivity of spot urine and 24-hour urine for neuroblastoma.

In centres associated with SIOPEN, HPLC/UPLC-MS/MS and HPLC-ED (high-performance liquid chromatography coupled with electrochemical detection) are most commonly used. In this study, we showed that HPLC-FD and UPLC-MS/MS have similar diagnostic sensitivities. Whether the latter is similarly interchangeable with HPLC-ED has

yet to be confirmed. The main advantage of using UPLC-MS/MS is improved speed providing rapid diagnostic results enabling to start the diagnostic workup earlier.²¹ Given the need for standardised measurements for future research, we have recently established the SIOPEX Catecholamine Working Group, aiming to harmonise the catecholamine metabolite analysis in different SIOPEX centres.

Our study has several limitations. First, this study was retrospective in nature with relatively small sample sizes, and our findings therefore require validation in a prospective cohort. Also, we did not measure catecholamine metabolites in 24-hour urine and spot urine within the same patients, so comparison between both sampling methods was only possible between different patients. Lastly, differences in sample handling and storage may have led to differences in catecholamine concentrations. However, by showing that metabolite concentrations measured by HPLC-FD and UPLC-MS/MS in a single batch showed a strong correlation, we believed these differences were minimal.

In conclusion, spot urine analysis can be used for all eight catecholamine metabolites without compromising their diagnostic sensitivities. Moreover, the diagnostic panel containing eight catecholamine metabolites has excellent diagnostic sensitivity in spot urine, which may have improved the diagnostic sensitivity compared to more limited catecholamine panels. Therefore, the Catecholamine Working Group recommends that the analysis of catecholamine metabolites, as part of neuroblastoma diagnostics, should be performed in a patient-friendly manner using spot urine sampling instead of 24-hour urine collection.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Villa Joep Foundation.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Matser YAH, Verly IRN, van der Ham M, et al. Optimising urinary catecholamine metabolite diagnostics for neuroblastoma. *Pediatr Blood Cancer*. 2023;70:e30289. <https://doi.org/10.1002/pbc.30289>