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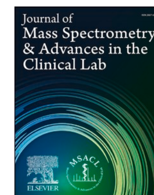

Research Article

# Validation of an LC-MS/MS method for urinary homovanillic and vanillylmandelic ACIDS and application to the diagnosis of neuroblastoma

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## ABSTRACT

*Background:* Urinary catecholamine metabolites are well-established biomarkers for neuroblastoma (NB). Homovanillic acid (HVA) and vanillylmandelic acid (VMA) are the most frequently measured metabolites within SIOPEN – Catecholamine Working Group laboratories. Here, we evaluated the performance of a new LC-MS/MS in vitro diagnostic (IVD) kit for HVA and VMA to facilitate inter-laboratory harmonization.
*Methods:* HVA and VMA and their deuterated internal standards were analyzed with a commercial method, on a ThermoFisher Quantiva LC-MS/MS. Validation was performed first using internal quality control and external quality assessment (IQC and EQA) samples. Next by clinical validation on 120 samples, previously tested by HPLC-ECD. Finally, 36 samples were exchanged between SIOPEN reference laboratories and analyzed by three methods.
*Results:* Using QCs and EQA the method was validated in a wide calibration range (4.61–830 μmol/L for HVA and 4.44–800 μmol/L for VMA). Intra-day CVs ($n = 5$) were 7 and 8 % for HVA and 5 and 6 % for VMA for QC low and QC high, respectively; Inter-day CV% were 7 and 3 % for HVA and 2 and 7 % for VMA at QC low and QC high, respectively. Its application to 120 clinical samples confirmed a high diagnostic accuracy. The inter-laboratory quality control assessment showed interchangeable results ($p = 0{,}73$ and $p = 0.15$ for HVA and VMA, respectively).

*Abbreviations:* AUC, Area Under ROC Curve; CI, Confidence Interval; Cr, Creatinine; CV, Coefficient of Variation; DA, Dopamine; E, Epinephrine; ECD, Electrochemical Detection; EQA, External Quality Assessment; ESI, Electrospray Ionization Source; HPLC, High-Performance Liquid Chromatography; HVA, Homovanillic Acid; ICH, International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; IQCs, Internal Quality Controls; IS, Internal Standard; IVD, In Vitro Diagnostic; LoA, Limits of Agreement; LC-MS/MS, Liquid Chromatography-tandem Mass Spectrometry; LLOQ, Lower Limit of Quantification; MN, Metanephrine; MRM, Multiple Reaction Monitoring; NB, Neuroblastoma; NE, Norepinephrine; NMN, Normetanephrine; PCTL, pertentile; QC, Quality Control; ROC, Receiver Operating Characteristic; SD, Standard Deviation; SIOPEN, International Society of Pediatric Oncology Europe Neuroblastoma Group; SPE, Solid-Phase Extraction; TSQ, Triple-Stage Quadrupole; UK NEQAS, United Kingdom External Quality Assessment Service; UPLC, Ultra-Performance Liquid Chromatography; VMA, Vanillylmandelic Acid; 3MT, 3-methoxytyramine.

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*Conclusion:* The LC-MS/MS IVD method could be considered a useful tool for clinical laboratories involved in the measurement of catecholamines, contributing to harmonization efforts.

## 1. Introduction

[1,2] accounting for 8–10 % of all childhood neoplasms. Diagnostic workup of NB involves the analysis of the catecholamine excretion profile, as catecholamine metabolites are increased in more than 90 % of patients affected by NB [3,4]. To date, the majority of clinical laboratories assess a restricted panel of catecholamine metabolites, often limited to the analysis of the phenolic acids, homovanillic acid (HVA) and vanillylmandelic acid (VMA), in urine of NB patients. The combined determination of these two metabolites has been shown to have a diagnostic sensitivity of 84 %, although it is now known that the extended panel, including all eight urinary catecholamine metabolites [dopamine (DA), epinephrine (E), norepinephrine (NE)] and metanephrines [3-methoxytyramine (3MT), metanephrine (MN), normetanephrine (NMN)], can increase the diagnostic sensitivity up to 95 % [5]. The European Neuroblastoma Research Network (SIOPEN) Catecholamine Working Group is promoting harmonization of catecholamine metabolite measurements among reference laboratories for biochemical diagnosis of NB with liquid chromatography-tandem mass spectrometry (LC-MS/MS) as the reference method. LC-MS/MS is considered the gold standard method for the quantification of small molecules in different fields of clinical biochemistry because it displays high accuracy and specificity starting from small sample volumes [6,7]. Nevertheless, high performance liquid chromatography (HPLC) coupled with electrochemical detection (ECD), being a cost-effective detection technique, is still widely used in clinical laboratories. However, ECD is laborious and time-consuming and lacks specificity compared to LC-MS/MS, thus the results obtained with this technique cannot be considered completely interchangeable with the results measured by LC-MS/MS [4,8]. This makes the harmonization process difficult by limiting the comparability of the results analyzed from different laboratories in large-scale clinical studies and the harmonization of measurements made for routine diagnostics. In this context, with the aim of aligning measurements, even in laboratories in which high-end mass spectrometers are not available, we validate a LC-MS/MS CE-IVD marked kit that is based on a simple and rapid extraction protocol with a derivatization step followed by solid-phase extraction (SPE), chromatographic separation and MRM detection in positive ion mode [4,8]. We have tested the method according to international guidelines [9] using LC-MS/MS, with a view to its application to clinical samples from NB patients, controls and EQA samples. Moreover, we have analyzed samples exchanged between reference laboratories as part of an inter-laboratory comparison within the SIOPEN Catecholamine Working Group.

## 2. Materials and methods

HVA and VMA were quantified by applying an in vitro diagnostic kit CE-IVD marked medical device: FloMass® VMA, HVA and 5-HIAA in Urine with online SPE (EUM24100, B.S.N. Biological Sales Network S.r.L. Cremona, Italy). The kit is based on online SPE, deuterated internal standards (IS) (VMA $^{13}C^{2}H_{3}$and HVA $^{13}C^{2}H_{3}$), a 6-level calibrator set (EUM19041, Biological Sales Network) (4.61, 23.0, 46.1, 138, 368, 830 µmol/L for HVA and 4.44, 22.1, 44.4, 133, 355, 800 µmol/L for VMA) and a 2-level Biogenic Amine control (EUM17051, Biological Sales Network). The lower limits of quantification (LLOQ) were 2.20 µmol/L and 1.51 µmol/L for HVA and VMA, respectively. The kit has been validated using an analytical column (EUM00C24, Biological Sales Network) coupled with a loading column (EUM00S02, Biological Sales Network). QCI and QCII concentrations were, respectively, 14.8, 349 µmol/L for HVA and 12.3, 317 µmol/L for VMA (batch dependent). Components for sample preparation included in the kit were: mobile phase A (500 mL), mobile phase B (300 mL), mobile phase C – SPE loading (1000 mL), stabilizing solution (120 mL), buffer solution (3 mL), reagent solution (3 mL) and IS solution (4.5 mL, stored at −20 °C). Internal quality controls (IQCs) were assayed at each analytical session by using 2-level Biogenic Amines Control. External quality assessment (EQA) samples from the International Proficiency Testing Scheme for urinary catecholamines and metabolites (UK NEQAS Birmingham Quality, Birmingham, UK) were performed monthly. Both IQCs and EQA samples were within the acceptance limits in the period analyzed.

### 2.2. Sample preparation

A 40 µL aliquot of urine samples, calibrators, or controls was added to 40 µL of IS Mix solution and 920 µL of stabilizing solution. Fifty µL of the obtained mix was then transferred to another vial. Twenty-five µL buffer solution, 25 µL reagent solution and 100 µL stabilizing solution were subsequently added. The samples were then vortexed, and centrifuged at 14,000×*g* for 5 min at 4 ∘C and the supernatant was transferred to autosampler vials and injected into the LC-MS/MS system.

### 2.3. Determination of HVA/VMA by LC-MS/MS – IVD kit

Analysis of HVA and VMA was performed at the Central Laboratory of Analyses of Giannina Gaslini Institute (Genoa, Italy), a tertiary care pediatric institute that is a reference center for biochemical diagnosis of NB in Italy and part of SIOPEN. HVA and VMA were quantified by LC-MS/MS using a TSQ Quantiva coupled to UHPLC Ultimate 3000 LC-MS/MS system (Thermofisher Scientific, Milan, Italy). Samples were prepared at room temperature following a derivatization step, extracted with an online solid phase extraction (online-SPE) and, ultimately transferred to the analytical column. LC-MS/MS analysis was carried out with the parameters reported in Supplementary Table 1.

### 2.4. Determination of HVA/VMA by HPLC-ECD assay

part of standard clinical procedures of analyses, VMA and HVA were measured in our laboratory by HPLC–ECD using an IVD-CE certified kit specifically intended for the analysis of these urinary metabolites (VMA, HVA, 5-HIAA in Urine – Chromsystems Instruments & Chemicals GmbH, Gräfelfing, Germany). Concentrations for the Calibrator Standard were 50.4 and 50.7 µmol/L for HVA and VMA, respectively. Additional urine controls, Endocrine Urine Control (product number 0040) with a normal-range concentration of 24.0 µmol/L for HVA and 21.5 µmol/L for VMA, and Endocrine Urine Control (product number 0050) with a pathological-range concentration of 70.8 µmol/L for HVA and 73.1 µ mol/L for VMA (both from Chromsystems Instruments & Chemicals GmbH, Gräfelfing, Germany), were assayed at each analytical run.

### 2.5. LC-MS/MS method partial validation

Since the analysis was performed using a CE-IVD certified kit for the determination of HVA and VMA, previously validated as a diagnostic tool and then applied to our LC-MS/MS system, we conducted a partial method validation in accordance with the International ICH guideline M10 on bioanalytical method validation and study sample analysis [9].

#### 2.5.1. Linearity, calibration curve and range

The $r^2$, slope and y-intercept, measured on the calculated values of

six calibrators from three independent series, were assessed to evaluate linearity. The calibration curves covered the concentration range 4.61–830 μmol/L for HVA and 4.44–800 μmol/L for VMA. The accuracy of each calibration standard was required to be within ±20 % of the nominal concentration at the LLOQ and within ±15 % at all the other levels as outlined in ICH guideline M10 on bioanalytical method validation and analysis of study samples [9].

#### 2.5.2. Precision, accuracy and LLOQ

Intra-day and inter-day precision and accuracy were determined at two concentration levels by analyzing the two IQC samples five times each in three different analytical runs over two weeks. Accuracy was expressed as the percent difference from the nominal value and precision as the coefficient of variation in percent (CV%). CV% was calculated as the ratio of the standard deviation to the mean. Precision and accuracy results were considered acceptable if ±15 % for each level and ±20 % for LLOQ. The LLOQ was defined as the lowest concentration measurable with a precision of ≤20 % and an accuracy of 80–120 % relative to the nominal concentration, along with a signal-to-noise ratio exceeding 5.

#### 2.5.3. Carryover

To assess carryover, four blank samples were analyzed after the analysis of the highest calibration standard point. Carryover was considered negligible if the signal was less than 20 % of the LLOQ and 5 % for the IS.

#### 2.5.4. Validation with EQA samples

To assess the performance of each method, external quality assessment (EQA) samples were used to evaluate trueness and accuracy. Ideally, EQA materials are commutable, meaning that the analytes and matrix of EQA materials and clinical samples should be interchangeable. The monthly EQA samples were analyzed using the HPLC-ECD method in the same analytical session as the clinical samples, as part of routine practice, and subsequently analyzed using the new LC-MS/MS method.

Analytical performance for specimens from distribution 271 is reported in Fig. 1. Specifically, the EQA samples used for validation were as follows: specimen 271A consisted of a mixture of real clinical specimens and was not spiked with exogenous compounds; specimen 271B was a pool of human urine with no added analytes; and specimen 271C was a pool of human urine with added analytes and some manipulation.

### 2.6. Clinical validation and method comparison: Application to clinical samples

For clinical validation, the LC-MS/MS method was compared to the HPLC-ECD assay currently used in our laboratory. The comparison was conducted on 120 clinical samples collected as first-morning spot urine, without dietary restrictions prior to collection. To preserve analytes and prevent volume changes, a few drops of concentrated hydrochloric acid (10 N HCl) were added to acidify the samples, ensuring a final pH of approximately 2–3 for long-term stability. The fresh urine samples were immediately analyzed using HPLC-ECD. After analysis, the samples were transferred to cryovials and stored at −80 °C with minimal light exposure to reduce potential analyte degradation.

All 120 urine specimens, previously analyzed using the HPLC-ECD method, were thawed and re-tested with the LC-MS/MS method. The samples were derived from pediatric subjects aged <18 months to 8 years.

Sixty samples were from patients with histologically confirmed NB, diagnosed between February 2020 and March 2023. Of these, 44 were obtained at disease onset, while the remaining 16 were from patients undergoing treatment (n = 10), in follow-up (n = 4), or experiencing relapse (n = 2).

The remaining 60 samples constituted the control group, consisting of patients in whom NB was suspected and catecholamine metabolites were measured as part of the diagnostic work-up. However, NB was not confirmed through imaging or histological analysis.

Clinical and demographic data of NB patients included in the study were obtained from the Italian NB Registry. Written consent for sample collection and the use of clinical and non-genetic data for research was provided by the patients' guardians. The study was approved by the Regional Ethical Committee (ANTECER Neuroblastoma, 16/09/2019).

HVA and VMA concentrations were expressed as a ratio to creatinine excretion, and results were considered elevated when concentrations exceeded the upper limit (95th percentile) of the age-specific reference range [10].

Additionally, as part of our study, 36 urine samples were analyzed in an inter-laboratory quality assessment using both the HPLC-ECD and LC-MS/MS methods. The specimens included in this cross-laboratory evaluation were from patients with a histologically confirmed diagnosis of NB.

Samples were obtained as part of standard care and collected as spot urine at the Wilhelmina Children's Hospital (Utrecht, The Netherlands). After routine analysis, they were exchanged with our laboratory. No dietary restrictions were imposed before sample collection. To minimize potential analyte degradation, urine samples were protected from light upon collection; however, acidification was not performed. All samples were stored at −20 °C after analysis.

Our results were compared with those from the biochemistry laboratory of the Princess Máxima Center for Pediatric Oncology (Utrecht, The Netherlands) as part of the SIOPEN catecholamine working group. Their analysis was conducted using ultra-performance liquid chromatography coupled with electrospray tandem mass spectrometry (UPLC-MS/MS) with an Acquity UPLC(H) combined with a Xevo TQ-XS (Waters, Etten Leur, The Netherlands).

The medical ethics committees of the AUMC (Reference number: W16_093#16.112) and the Princess Máxima Center (Reference number: PMCLAB2019.075) determined that the Medical Research Involving Human Subjects Act does not apply to this study.

| | Specimen | Pool | Result | Target | Specimen %bias | A score | B score | C score | A | B | C |
|---|---|---|---|---|---|---|---|---|---|---|---|
| VMA [HMMA] (umol/24h) | 271A | C452 | 14.64 | 16.9 | -13.3 ♦ | 51 | -4.7 | 6.3 | ●↔ | ●↔ | ●↔ |
| | 271B | C436 | 14.64 | 14.3 | +2.0 ♦ | | | | | | |
| | 271C | C418 | 144.86 | 165 | -12.2 ♦ | | | | | | |
| HVA (umol/24h) | 271A | C452 | 15.37 | 16.3 | -5.6 ♦ | 41 | +2.1 | 5.1 | ●↔ | ●↔ | ●↔ |
| | 271B | C436 | 24.7 | 24.6 | +0.2 ♦ | | | | | | |
| | 271C | C418 | 87.28 | 88 | -0.8 ♦ | | | | | | |

**Fig. 1.** From UK NEQUAS report for Urinary Catecholamines & Metabolites. Analytical Performance for specimens from distribution 271(EQA). The green traffic light reflects that the laboratory is performing as well as the state-of-the-art allows. The urine volume has been assumed to be 1.0L, collected over a 24 h period, to avoid there being confusion between those laboratories that report in units/L and those that report in units/24 h. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### 2.7. Data analysis and statistics

Method comparison analyses were performed using MedCalc® software (MedCalc Software Ltd., Ostend, Belgium) with Passing-Bablok regression analysis [11]. The 95 % confidence intervals (CI) for the slope and intercept were determined.

To assess the relative differences between the three methods, the Bland–Altman test was used, plotting the differences against the mean value for all three methods. The mean relative differences and the 1.96 standard deviations (SD) of the differences were calculated.

To evaluate the diagnostic sensitivity and specificity of the HPLC-ECD assay and the new LC-MS/MS method, Receiver Operating Characteristic (ROC) curve analysis was performed. The area under the ROC curve, along with its 95 % confidence interval (CI), was calculated using the Mann-Whitney *U* test.

## 3. Results

### 3.1. LC-MS/MS method partial validation

The LLOQ was 2.20 μmol/L for HVA and 1.51 μmol/L for VMA. A weighted (1/x) quadratic regression model was used for the calibration curve. The mean calibration curve equations were: $y = -0.0362259 + 0.0177121*X - 2.32627\ e\text{-}6X^2$ $R^2 = 0.9993$ W:1/X for HVA and $y = -0.0146781 + 0.0191058*X$ ($R^2 = 0.9992$, W:1/X) for VMA (Fig. 2).

A linear relationship was observed between the analyte peak area and the corresponding concentration across the entire concentration range ($R^2 = 0.99$). The HVA and VMA concentration values obtained did not deviate significantly from the nominal values (±15 %). Inter-day and intra-day accuracy and precision are presented in Table 1.

Carry-over was negligible (<15 % for both HVA and VMA). Results from the analysis of the EQA samples fell within the acceptance range for both HVA and VMA, as measured by HPLC-ECD and LC-MS/MS.

### 3.2. Clinical validation

#### 3.2.1. Analyses of clinical samples

Results for urinary HVA and VMA concentrations from the 120 clinical samples were initially expressed as ratios to creatinine (Cr) concentration (HVA/Cr and VMA/Cr) in μg/mg to align with the age-specific reference ranges for urinary HVA and VMA, previously established in our laboratory [10]. To ensure consistency throughout the study, the measurements were then converted to μmol/mmol creatinine. The concentrations spanned a wide range: 1.24–685.52 μmol/mmol Cr for HVA (by HPLC-ECD), 0.68 – 538.44 μmol/mmol Cr for HVA (by LC-MS/MS), 1.22 – 575.10 μmol/mmol Cr for VMA (by HPLC-ECD), and 0.48 – 665.03 μmol/mmol Cr for VMA (by LC-MS/MS). Creatinine values were previously measured as part of the routine analysis. The comparison of the results of the two methods is shown in Supplementary Figs. S1 A, B, C, and D, showing the Bland-Altman plot test and the non-parametric Passing-Bablok regression analyses. The Bland-Altman plot exhibits a significant bias between the HPLC-ECD method and the new

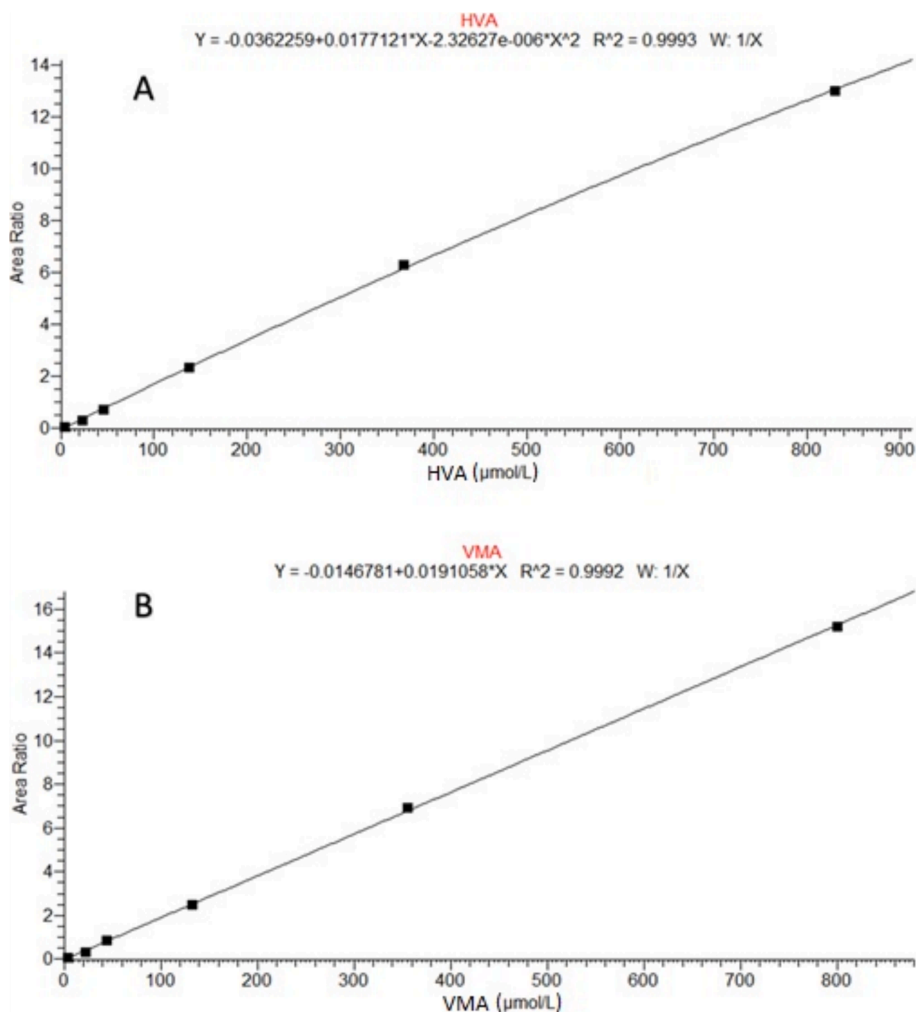


**Fig. 2.** Six-point mean calibration curve for HVA and VMA in urine. The $r^2$, slope and y-intercept were measured on three independent calibration curves. (Area Ratio, peak area of analyte /peak area of IS).

**Table 1**
Results of inter-day (n = 5 x 3) and intra-day ($n = 5$) accuracy and precision (expressed as CV%) for HVA and VMA. SD is standard deviation.

| HVA | Nominal Value (µmol/L) | Inter-day SD | Inter-day Precision (CV %) | Inter-day Accuracy (bias %) | Intra-day SD | Intra-day Precision (CV %) | Intra-day Accuracy (bias %) |
|---|---|---|---|---|---|---|---|
| **QC I** | 14.8 | 1.10 | 7 % | 103 % | 1.06 | 7 % | 110 % |
| **QC II** | 349 | 10.60 | 3 % | 100 % | 29.99 | 8 % | 106 % |

| VMA | Nominal Value (µmol/L) | Inter-day SD | Inter-day Precision (CV %) | Inter-day Accuracy (bias %) | Intra-day SD | Intra-day Precision (CV %) | Intra-day Accuracy (bias %) |
|---|---|---|---|---|---|---|---|
| **QC I** | 12.3 | 0.30 | 2 % | 104 % | 0.71 | 5 % | 110 % |
| **QC II** | 317 | 24.38 | 7 % | 104 % | 19.53 | 6 % | 107 % |

LC-MS/MS method, with p-value ≤ 0.05 and a mean difference of 26.7 (95 % Confidence Interval: 15.8 to 37.5) and 13.2 (95 % Confidence Interval: 4.6 to 21.8) for the assessed measurements of HVA and VMA, respectively. The interval between the lower and upper limit of agreement (−90.9 to 144.2 and −79.8 to 106.2 ± 1.96 SD for HVA and VMA, respectively) includes the 95 % of differences between the methods. Measurements outside this interval do not compromise the diagnostic efficacy of either method. The Passing-Bablok regression equation, Intercept and Slope were as follows:

HVA: $y = 0.0737533 + 0.582893\,x$ ; 0.07375 (95 % CI: −0.2575 to 0.3042); 0.5829 (95 % CI: 0.5352 to 0.6174).

VMA: $y = -0.200023 + 0.666971\,x$ ; −0.2000 (95 % CI: −0.4605 to 0.1023); 0.6670 (95 % CI: 0.5823 to 0.7179).

The analysis indicated the presence of a proportional bias. Nevertheless, these differences should be interpreted based on the established diagnostic criteria of acceptability. Indeed, it is possible that small proportional or constant systematic errors, identified by the regression analysis, while statistically significant, do not preclude the use of the method [12].

The diagnostic performance of the two methods was assessed by calculating sensitivity and specificity through ROC curve analysis on values obtained from 44 patients at the onset of disease and 60 controls (supplementary Fig. S2 A, B, C, and D). The Youden Index was applied in order to obtain an optimal threshold for each analyte. As listed in Table 2, the calculated AUCs reflect the overall performance of the two methods. Both methods displayed a similar performance in terms of sensitivity and specificity. Thresholds for the AUC/sensitivity/specificity analysis were >12.01 and >6.12 for HVA and VMA, respectively, by HPLC-ECD and >12.11 and > >5.19 for HVA and VMA, respectively, by LC-MS/MS. Despite some discrepancies, for the 44 samples at the disease onset, the diagnosis of neuroblastoma was confirmed by both methods and clinical agreement with data was achieved for these samples.

Quantitative results aligned with our age-specific reference ranges for urinary HVA and VMA [10], which was applied to both methods: very high values of HVA and VMA, measured and compared with each other, were found to be in agreement in relation to their clinical significance allowing the diagnosis of NB to be confirmed by either method in all patients at the onset of the disease.

As for the remaining NB patients, discrepant quantitative results were obtained for HVA or VMA in 14 out of 16 cases when using our age-specific reference ranges, with values obtained by HPLC-ECD higher than those obtained by LC-MS/MS. This may potentially lead to a different clinical interpretation and a misclassification between positive or negative results. Of note, these patients were all under treatment or in follow-up and, in light of other examinations (such as electrocardiogram − ECG, complete blood count − CBC, blood chemistry tests and diagnostic imaging tests), were all classified as disease-free or in remission. Both methods correctly identified the NB disease status of the two patients at relapse, showing positive results for HVA and VMA.

Data analysed against our reference ranges are summarized in Table 3. All quantitative data are shown in Supplementary Table 2 (expressed as µg/mg Cr) and 3 (expressed as µmol/mmol Cr).

Since the pre-analytical steps for both HPLC-ECD and LC-MS/MS assay were the same, the stability of the analytes in acidified urine stored at −80 °C was not evaluated. The addition of HCl to urine to prevent the degradation of HVA and VMA is a well-established and widely accepted practice [13–19]. However, a partial degradation of the samples at the time of measurements by the new method cannot be excluded, which may also explain the discrepancy found between the values measured by the two methods.

#### 1.2.2. *Inter-laboratory comparison*

Statistical analysis was performed on the set of 36 urine samples of the inter-laboratory comparison. The three methods were compared *two-by-two* to identify which one correlated best between the methods to achieve harmonization. The results of the comparison of the three methods are shown in Fig. 3 (A and B), as Bland-Altman plot test, and in Fig. 4 (A and B), as non-parametric Passing-Bablok regression analyses. In the two figures, comparing the HPLC-ECD method with both LC-MS/MS methods, a sample with a very high VMA value can be observed. The large negative difference between the two methods is due to the fact that for this sample, with a high concentration of the analyte, analysis by HPLC-ECD reveals a poor linear range of the detector in high concentrations. Urinary HVA and VMA concentrations were expressed as µmol/L. Bland-Altman analysis for HVA and VMA concentrations are listed in Supplementary Table 4. Results, obtained by both LC-MS/MS methods, compared with results obtained by HPLC-ECD, showed the presence of significant bias in the Bland-Altman analysis for HVA values ($p = 0.0048$ for HPLC-ECD and LC-MS/MS performed by TSQ Quantiva; $p = 0.0230$ for HPLC-ECD and UPLC-MS/MS performed by Xevo TQ-XS) and proportional bias in the Passing-Bablok analysis (Supplementary Table 5)

**Table 2**
Diagnostic performance of HPLC-ECD and LC-MS/MS. Area under the ROC curves (AUC) with 95% confidence interval (CI) and sensitivity and specificity with 95% confidence interval (CI) are reported for HVA and VMA measurements.

| | AUC | 95 % CI | SENSITIVITY (%) | 95 % CI | SPECIFICITY (%) | 95 % CI | Threshold (µmol/L Cr) |
|---|---|---|---|---|---|---|---|
| **HVA** HPLC-ECD | 0.998 | 0.961 to 1.0 | 95.5 | 84.5 – 99.4 | 100 | 94.0 – 100.0 | >12.01 |
| **HVA** LC-MS/MS | 0.997 | 0.960 to 1.0 | 95.5 | 84.5 –99.4 | 100 | 94.0 – 100.0 | >12.11 |
| **VMA** HPLC-ECD | 0.998 | 0.962 to 1.0 | 100 | 92.0 – 100.0 | 96.7 | 88.5 – 99.6 | >6.12 |
| **VMA** LC-MS/MS | 0.999 | 0.964 to 1.0 | 100 | 92.0 – 100.0 | 98.3 | 91.1 – 100.0 | >5.19 |

**Table 3**
Summary of positive and negative measurements obtained from the analysis of the 60 NB samples by HPLC-ECD and LC-MS/MS methods for quantification of HVA /Cr and VMA /Cr (mg/g Cr) against our age-specific reference ranges [10] in relation to the 95 th percentiles (PCTL).

| | HPLC-ECD vs LC-MS/MS | | | | | HPLC-ECD vs LC-MS/MS | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **HVA/Cr** | | **HVA/Cr** | | | **VMA/Cr** | | **VMA/Cr** | | |
| Age | **Sample no** | **Value** | **Sample no** | **Value** | **Range 95thPCTL** | **Sample no** | **Value** | **Sample no** | **Value** | **Range 95th PCTL** |
| 0–3 *m* | 7 | (+) | 7 | (+) | 28.60 | 7 | (+) | 7 | (+) | 15.92 |
| 3 m-6 *m* | 5 | (+) | 41 | (+) (−) | 33.43 | 5 | (+) | 5 | (+) | 16.85 |
| 6–12 *m* | 6 | (+) | 51 | (+) (−) | 25.30 | 6 | (+) | 6 | (+) | 16.62 |
| 1-2a | 6 | (+) | 42 | (+) (−) | 21.85 | 6 | (+) | 51 | (+) (−) | 12.05 |
| 2-5a | 24 | (+) | 231 | (+) (−) | 18.62 | 24 | (+) | 186 | (+) (−) | 11.87 |
| 5-10a | 11 | (+) | 101 | (+) (−) | 10.70 | 11 | (+) | 92 | (+) (−) | 8.40 |
| 10-15a | 1 | (+) | 1 | (−) | 9.35 | 1 | (+) | 1 | (+) | 9.16 |

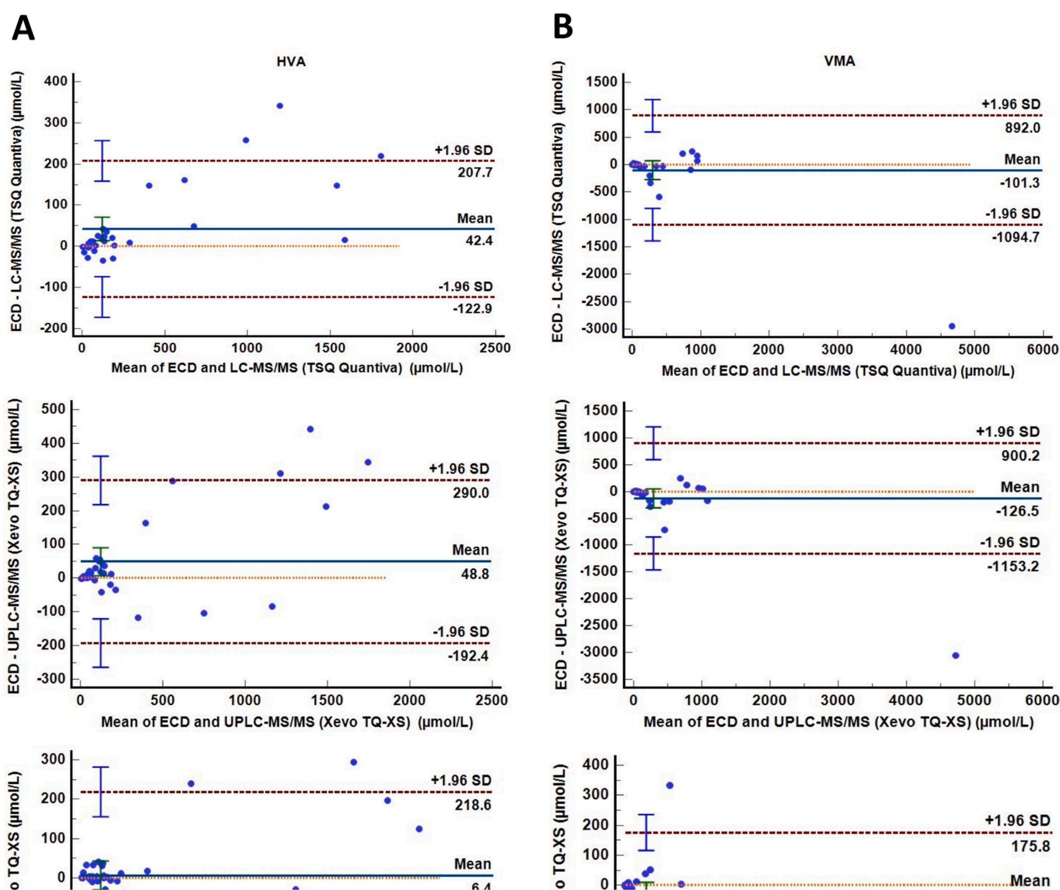


**Fig. 3.** **A** and **B**: Bland–Altman plot of inter-laboratory comparison between the reference method HPLC-ECD, the new LC-MS/MS method performed by TSQ Quantiva and Utrecht laboratory method UPLC-MS/MS performed by Xevo TQ-XS for HVA and VMA. The blue solid line indicates the mean difference (=bias) between the two methods. The brown dashed lines indicate the upper and lower 95 % LoA (= bias ± 1.96 × SD). The outlier in panel B, noticeable in the comparison between the ECD method and both LC-MS/MS methods, was a sample with a very high value for VMA. The large negative difference between the two methods is due to the fact that for this sample, with high analyte concentration, analysis by HPLC-ECD reveals a poor linear range of the detector in high concentrations. Results are expressed in µmol/L. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

for HVA and VMA values. On the other hand, the LC MS/MS method performed by TSQ Quantiva compared with the method performed by Xevo TQ-XS, exhibited a high correlation among the results obtained by making them interchangeable ($p = 0.7264$ and $p = 0.1500$ for HVA and VMA, respectively).

## 4. Discussion

To date, the gold standard method for the determination of urinary catecholamines is LC-MS/MS due to its merits of high selectivity, high sensitivity and high throughput [20,21]. The majority of the studies using this method for the measurement of the polar acids HVA and VMA describe a Dilute-and-Shoot approach [22,23]. This technique offers

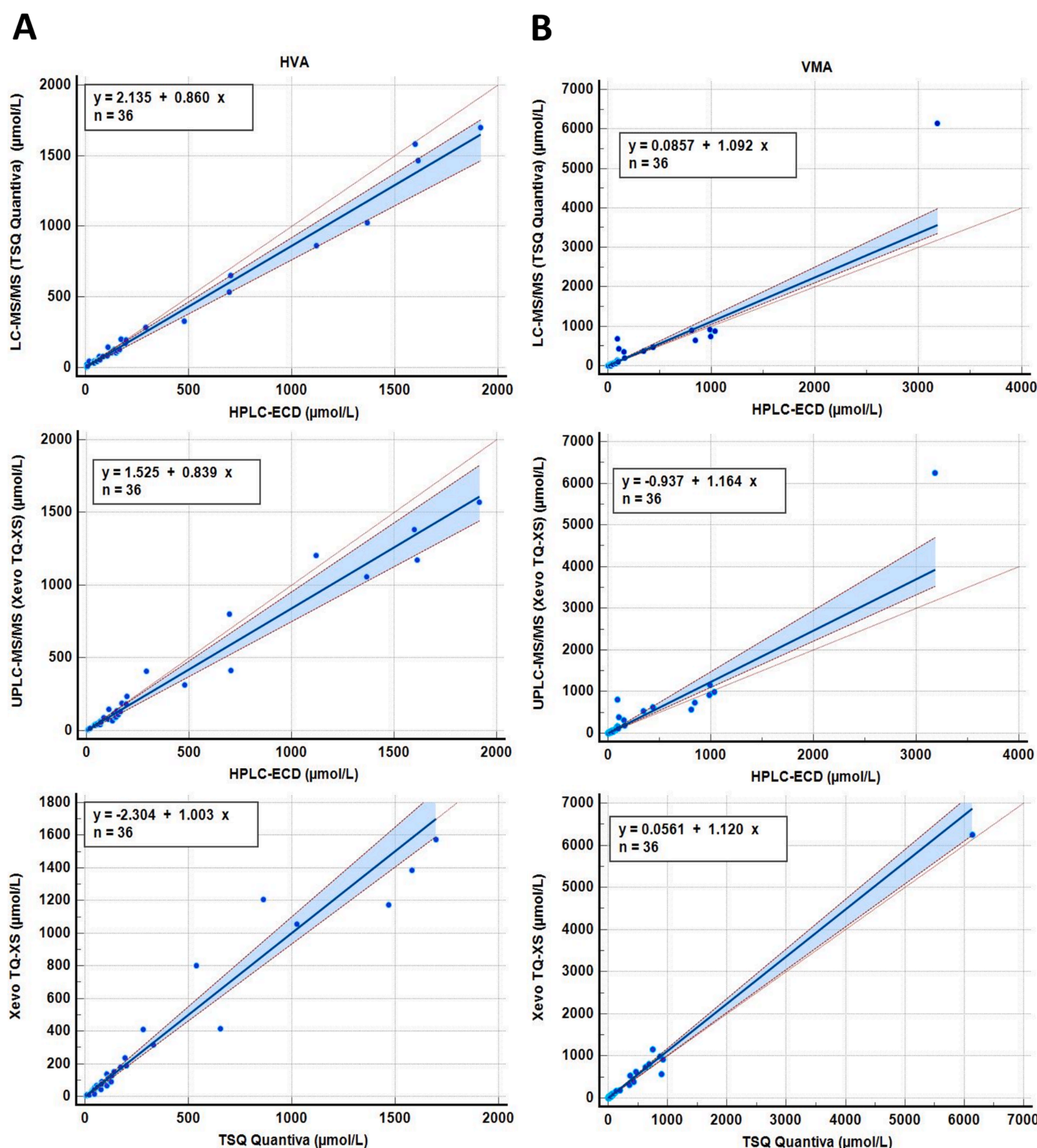


**Fig. 4.** **A** and **B**: Inter-laboratory comparison between the reference method HPLC-ECD, the new LC-MS/MS method performed by TSQ Quantiva and Utrecht laboratory method UPLC-MS/MS performed by Xevo TQ-XS for HVA and VMA. In the Passing–Bablok regression, the red dotted line indicates the regression in case of perfect agreement. The blue solid line represents the actual regression obtained by the comparison of HPLC-ECD and LC-MS/MS methods. The brown dashed lines represent the 95% CI around the obtained regression. The outlier in panel B, noticeable in the comparison between the ECD method and both LC-MS/MS methods, was a sample with a very high value for VMA. The large negative difference between the two methods is due to the fact that for this sample, with high analyte concentration, analysis by HPLC-ECD reveals a poor linear range of the detector in high concentrations. Results are expressed in μmol/L. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

several advantages, including simplicity, minimal and rapid sample preparation, reduced solvent consumption, cost-effectiveness, and lower waste production. Yet, issues with the repeatability, reproducibility, accuracy and precision may occur with this assay. The main drawbacks of this technique include matrix dilution, which can lead to a higher limit of detection and the potential dilution of the analytes [24]. The HPLC-ECD assay has so far been used as a routine diagnostic method for the analysis of urinary HVA and VMA in our laboratory. However, this technique experiences several disadvantages. These challenges include complex and time-consuming sample preparation, requiring SPE offline in-sample cleanup columns; the need for well-trained operators; an injection-to-injection time of approximately 20 min; and typical back-pressure issues, which can be either too high or too low, potentially affecting the flow rate. In addition, HPLC-ECD exhibits lower specificity, relying on the redox capabilities of the catechol group, in contrast to MS detection, which is predicated on the mass/charge ($m/z$) ratio [25]. For these reasons, and with the aim of pursuing a process of harmonization between methods, the LC-MS/MS method performed by TSQ Quantiva was applied in our laboratory to measure clinical samples.

Herein, we have presented a new LC-MS/MS method that includes a derivatization step, on-line SPE and ionization using ESI in a positive mode. The derivatization step functionalizes the molecules, improving the ionization yield thereby ensuring greater applicability of the method, even on mid-range spectrometers. Derivatization reagents for the analysis of small molecular weight compounds in complex matrices, such as urine, may improve the detection sensitivity, selectivity, chromatographic separation and identification capability of LC-MS/MS analysis. In particular, the derivatization of polar compounds, such as the polar acids HVA and VMA, increased their volatility, thermal stability and detection sensitivity. An additional advantage of the method is

the use of online SPE, which prevents contamination of the ion source and the accumulation of retained compounds on the LC column, issues that often arise with direct urine injection [26,27]. This feature enables fast and automated analytical performance. The new method could broaden the application of LC-MS/MS for HVA and VMA measurement in clinical laboratories, thus facilitating the harmonization process. Moreover, it reduces the runtime to 8.5 min and maximizes sample throughput.

LC-MS/MS applications in routine clinical laboratory require thorough method validation and integration with established procedures and programs to ensure quality assurance. EQA results for all samples were within the acceptance limits (±25 %), as confirmed by UK NEQAS evaluation- report. The report indicated the deviation of the results from the assigned target values, certifying that the laboratory's results met the specified analytical performance criteria. No clinical misinterpretation was identified in the HPLC-ECD analysis of the 120 clinical samples. All patients with elevated values (44 samples) were correctly diagnosed with neuroblastoma. However, discrepancies were observed when comparing results from LC-MS/MS analysis performed on a TSQ Quantiva system, which showed quantitative discordance with those obtained by the HPLC-ECD method. Consequently, quantitative harmonization between the two methods was not achieved. To address this, more accurate reference intervals are being established to align with the new LC-MS/MS method. Importantly, no clinical discrepancies were observed when comparing the three methods on the 36 anonymized samplesall of which showed values consistent with reference ranges and previous neuroblastoma diagnoses, confirming clinical correlation across methods.

Moreover, LC-MS/MS results obtained using the TSQ Quantiva system were quantitatively comparable to those from the reference laboratory using the Xevo TQ-XS system, demonstrating strong agreement and suggesting potential harmonization between laboratories. Most importantly, both methods confirmed the clinical significance of the results, indicating that they can be considered interchangeable in a clinical setting.

HVA and VMA are the most commonly measured metabolites in the diagnosis of NB. Extending the analysis to the remaining six urinary catecholamines and metanephrines (DA, E, NE, MN, NMN, and 3MT) using the same LC-MS/MS approach is feasible but remains a work in progress. Implementing a complete panel of eight metabolites could not only improve diagnostic accuracy to approximately 95 %, but also represent a significant step toward harmonizing results across laboratories.

## 5. Conclusion

The results obtained using this new LC-MS/MS method are comparable to those from the Utrecht reference laboratory's UPLC-MS/MS analysis. Despite the inability to achieve quantitative harmonization with HPLC-ECD, no clinical discrepancies were observed. This new approach has proven to be a reliable tool for accurately measuring HVA and VMA, supporting harmonization efforts among laboratories involved in the biochemical diagnosis of NB.

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## CRediT authorship contribution statement

**Lucilla Rossi:** Writing – original draft, Formal analysis. **Yvette A.H. Matser:** Writing – review & editing, Resources. **Sebastiano Barco:** Writing – review & editing, Visualization, Supervision, Software, Formal analysis. **Alessia Cafaro:** Writing – review & editing, Writing – original draft, Visualization. **Federica Pigliasco:** Visualization. **Margherita Biondi:** Visualization, Formal analysis. **Fabrizio Mancin:** Writing – review & editing. **Maria van der Ham:** Writing – review & editing, Resources. **Monique G.M. de Sain-van der Velden:** Writing – review & editing, Resources. **Shifra Ash:** Writing – review & editing, Resources. **Maja Beck Popovic:** Writing – review & editing, Resources. **André B.P. van Kuilenburg:** Writing – review & editing, Resources. **Massimo Conte:** Writing – review & editing, Resources. **Alberto Garaventa:** Writing – review & editing, Resources. **Godelieve A.M. Tytgat:** Writing – review & editing, Resources. **Giuliana Cangemi:** Writing – review & editing, Supervision, Resources, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmsacl.2025.04.007.

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