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# Performances of a multidimensional on-line SPE-LC-ECD method for the determination of three major catecholamines in native human urine: Validation, risk and uncertainty assessments

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**Abstract**

A novel, multidimensional on-line SPE-LC method with electrochemical detection is described for the fully automated and direct analysis of the catecholamines norepinephrine, epinephrine and dopamine in urine. The integrated extractive clean-up of the raw biofluid is based on a SPE-column packed with restricted access material (RAM) which is modified with the affinity ligand nitrophenylboronic acid. The method was fully validated according to a recent approach based on an accuracy profile. The acceptance limits were set at ±15% of the nominal concentration values. The method was found accurate over a concentration range from 15 to 500 μg/l for norepinephrine, from 5 to 500 μg/l for epinephrine and from 50 to 500 μg/l for dopamine. The relative risk for the use of the validated method in routine analysis was also assessed based on this validation strategy. It was found that at most 3.5% of future sample measurements will fall outside the acceptance limits. This demonstrates the high reliability of the analytical method described. Moreover, the measurements uncertainties were deduced from the validation experiments without any additional effort.


*Keywords:* Catecholamines; Norepinephrine; Epinephrine; Dopamine; Urine; On-line SPE; Restricted access materials; RAM; Multidimensional; ECD; Column-switching; Validation; Accuracy profile; Risk analysis; Measurement uncertainty

## 1. Introduction

Epinephrine (E), norepinephrine (NE) and dopamine (DA, cf. Fig. 1) represent endogenous catecholamines which act as hormones and/or neurotransmitters. These biogenic amines serve as tumor markers in 24 h urine sample of individuals screened and/or monitored for neuroblastoma or pheochromocytoma in humans [1]. Consequently, there is a need to have a reliable bioanalytical method available for the routine clinical-chemical extraction, separation and quantitation of these diagnostic marker molecules. Many papers have been published, describing methods for LC separation and detection of catecholamines in biological fluids, such as urine or plasma. Catecholamines are electroactive compounds and therefore can be quantitated using an electrochemical detector [2–11]. Various methods have been described for pretreatment of the complex biological matrices to be analyzed as well as for the extraction of trace amounts of catecholamines. These include liquid–liquid extraction [12,13], adsorption on aluminum oxide [13–15], off-line and on-line solid-phase extraction (SPE) using cation-exchange packings [9,16,17] or diphenylborate as complexation reagent [10,18–20] and phenylboronic acid as immobilized affinity ligand for on-line SPE [21–25]. The main limitations with liquid–liquid extraction, alumina adsorption or cation exchange packings are that they are not selective enough and that they do not give high extraction yields. Furthermore, extraction on alumina as well as off-line solid phase extraction requires complex washing procedures which introduce variability in

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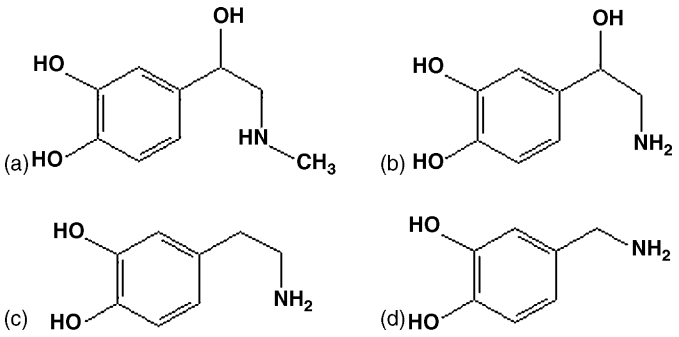


Fig. 1. Chemical structures of: (a) epinephrine, E; (b) norepinephrine, NE; (c) dopamine, DA; (d) dihydroxybenzylamine (internal standard).

recovery. All these sample purification methods are complex, time consuming or labour intensive. To increase the efficiency of the sample preparation step, an alternative is to use highly selective ligand such as boronic acid derivative which selectively sorbs the *cis*-diol group present in catecholamines transmitter. In addition, the combination with restricted access material and automated on-line extraction methods increases the reliability of the results. To our knowledge no such on-line SPE method has been described which uses electrochemical detection for the quantitation of urinary catecholamines.

To ensure that the analytical method can fulfill its objective, which is to quantify the three major catecholamines in urine, the method has to be validated. For that purpose, an original strategy proposed by a Commission of the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP) was applied which is based on accuracy profiles as a decision tool [26,27]. This approach is also used to select the most appropriate response function, to estimate the limit(s) of quantitation and to evaluate the concentration range. Furthermore, this strategy allows to estimate and control the risk associated with the future use of the method [26–29]. However, to allow an adequate interpretation and comparison of the results to toxics or pathological levels or to regulatory acceptance limits, validation is not enough; the uncertainty related to the measurements should be estimated. Without any further experiments it was possible to estimate the uncertainty of measurements using the original validation strategy.

## 2. Experimental

### 2.1. Chemicals

Norepinephrine bitartrate salt, epinephrine and dopamine hydrochloride were obtained from Sigma (Steinheim, Germany), 3,4-dihydroxybenzylamine hydrobromide was obtained from Fluka (Steinheim, Germany). Octane-1-sulfonic acid sodium salt, potassium di-hydrogen phosphate and di-ammonium hydrogen phosphate were all of analytical grade and were purchased from Merck (Darmstadt, Germany). EDTA disodium salt dehydrate, also of analytical grade, was purchased from Fluka (Steinheim, Germany). Sodium hydroxide, hydrochloric acid (Titrisol 1 N) and phosphoric acid (85%) were obtained from Merck (Darmstadt, Germany). Water, acetonitrile and methanol were of HPLC grade and purchased from J.T. Baker (Deventer, The Netherlands).

### 2.2. Apparatus

A Summit ×2 dual gradient HPLC system (Dionex, Idstein, Germany) equipped with: a solvent rack model SOR-100, two low-pressure gradient pumps with integrated on-line degasser P680, an automated sampler injector ASI-100 and a thermostated column oven TCC-100 was used. The Chromeleon® V6.60 manager software (Dionex) was loaded for the control of the analytical system and data collection. The electrochemical detector (EC3000), the detector cell (EC4000) and the thermostat (HT3000) were from Recipe Chemicals & Instruments GmbH, Munich, Germany.

A three way low pressure switching valve (Recipe) was placed between the outlet of the analytical column and the electrochemical cell and controlled automatically by the LC-system.

The SPE-column (CAT-PBA, 30 mm × 4 mm ID, Recipe Chemicals & Instruments GmbH, Munich, Germany) contains a restricted access material (RAM) modified with the affinity ligand nitrophenylboronic acid. The size exclusion limit of the RAM SPE-column amounts to approximately 10 kDa. The analytical column (150 mm × 4.6 mm ID; particle size: 5 μm) was packed with a reversed phase material and obtained from Recipe Chemicals & Instruments GmbH (Munich, Germany). The SPE-column and the analytical column were coupled via the ten-port switching valve integrated in the column oven of the Summit ×2 dual gradient HPLC system, whereby two adjacent ports were sealed. The e.noval® software V2.0 (Arlenda, Liège, Belgium) was used to compute the accuracy profiles and validation results.

### 2.3. Standard solutions

Stock solutions of the catecholamines investigated were prepared by dissolving an accurately weighed amount of the analytes in 0.1 M HCl. These solutions were stored at 4 °C and newly prepared each day except for the internal standard which was used for one week. The stock solutions of catecholamines were diluted with HCl (0.1 M) and used to spike aliquots of 24 h human urine up to 5 concentration levels ($m = 5$) covering a range from 5 to 500 μg/l. Two types of spiked urine samples were prepared daily, namely calibration standards and validation standards, both having the same concentrations. Each sample was also spiked with internal standard in order to obtain a concentration of 100 μg/l. Each calibration standard was analyzed twice ($n = 2$) whereas each validation standard was analyzed three times ($n = 3$). Calibration and validation standards were prepared for five different days ($k = 5$).

### 2.4. Urine collection

Twenty-four hours human urine was collected under standardized conditions using a newly developed kit, Uriset24® (Sarstedt AG & Co., Nümbrecht, Germany) [30]. Aliquots were stored at −20 °C. Urine samples were thawed at room

Table 1
Valve positions, timing and flow rates

| Analysis step | Time (min) | Position high pressure SV | Position heart-cut 3 port SV | Mobile phase A (ml/min) | Mobile phase B (ml/min) |
|---|---|---|---|---|---|
| 1 | 0–2 | A | A | 2.0 | 1.2 |
| 2, 3, 4 | 2–3 | B | B | 2.0 | 1.8 |
| 3, 4 | 3–5.3 | A | B | 0.5 | 1.8 |
| 3, 4 | 5.3–5.5 | A | B | 0.5 | 1.8 |
| 3, 4 | 5.5–20 | A | A | 0.5 | 1.2 |

temperature and centrifuged at 12,000 × *g* for 15 min prior to analysis.

### 2.5. On-line SPE and analytical separation

A fully automated SPE-LC analysis cycle using the Summit ×2 dual gradient HPLC system consists in four distinct steps (Fig. 2):

1. Matrix depletion and analyte extraction
   The sample (standard solution or 20 μl of raw human urine) is loaded with mobile phase A (0.2 M diammoniumhydrogenphosphate, 3.72 g/l EDTA and methanol, 95:5, v/v, adjusted to pH 8.7) at a flow rate of 2.0 ml/min via the autosampler onto the SPE-column. While the sample matrix is flushed to waste within 2 min, urinary catecholamines are retained and extracted by chemospecific formation of a cyclic boronic ester [18]. Due to the formation of a reversible covalent bond with the affinity ligand, recovery of the target analytes and depletion of the sample matrix is quantitative.

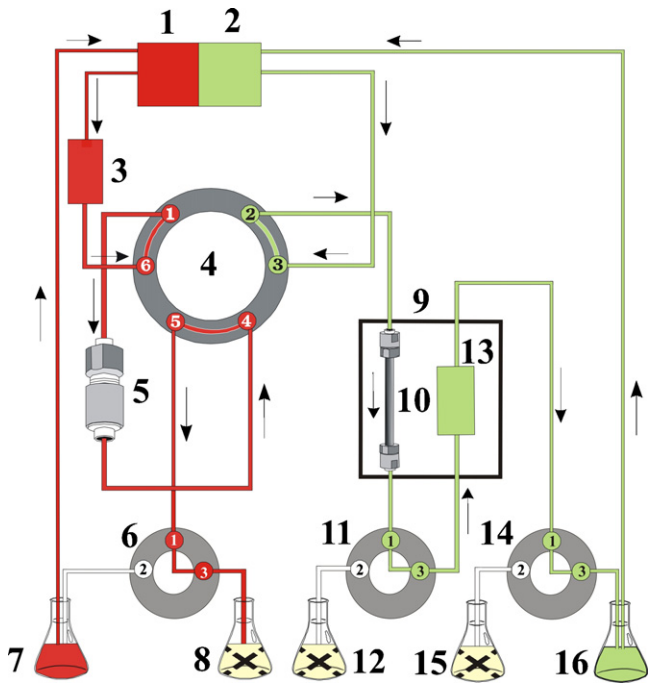


Fig. 2. Instrumental set-up of the SPE-LC-ECD system: (1) pump A; (2) pump B; (3) autosampler; (4) high-pressure-valve; (5) SPE-column; (6) low pressure valve; (7) mobile phase A; (8) waste; (9) thermostat; (10) analytical column; (11) heart-cut low pressure valve; (12) waste; (13) electrochemical cell; (14) low pressure valve; (15) waste; (16) mobile phase B.

2. Analyte transfer from SPE-column to analytical column
   By switching the automatic valve (cf. Table 1, 10-port SV), the SPE-column and the analytical HPLC column are series-connected. The mobile phase B (50 mM potassium dihydrogen phosphate, 2.5 mM sodium octylsulfonate, 0.1 g/l EDTA and acetonitrile, 96.5:3.5, v/v, adjusted with phosphoric acid to pH 3.5) which is delivered to the SPE-column in a back-flush mode hydrolyses the cyclic ester and transfers the released analytes on top of the analytical column within 1 min at a flow-rate of 1.8 ml/min.
3. Analyte separation
   After switching back into the original valve position (cf. Table 1, 10-port SV), the catecholamines are separated under isocratic conditions by ion-pair reversed phase HPLC within 20 min at a flow rate of 1.2 ml/min and 30 °C. The catecholamines were monitored electrochemically applying a working potential of 600 mV. In order to prevent any disturbance of the electrochemical cell due to the change of the composition of the mobile phase, the resulting plug composed of a buffer mixture was directed to waste (heart-cut; cf. Table 1, 3-port SV).
4. Reconditioning of the SPE-column
   During the analytical separation of the catecholamines the SPE-column is flushed with mobile phase A in order to regenerate it and to be ready for the next injection.

## 3. Results and discussion

### 3.1. Sample clean-up, analyte separation and detection

The described on-line SPE-LC-ECD method allows the injection of raw human urine, integrated extractive sample clean-up, ion-pair reversed phase separation and electrochemical detection of the catecholamines norepinephrine, epinephrine and dopamine.

The SPE step is chemoselective with regards to the analytes investigated. The immobilized affinity ligand nitrophenylboronic acid forms a reversible covalent bond with the catecholamines via their *cis*-diol substructure [18]. Three chromatographic modes are applied for the extraction and separation of the target analytes, namely affinity-, size-exclusion- and ion-pair-reversed phase chromatography. All this modes are orthogonal to each other. Thus, this on-line SPE-LC method is multidimensional and for this reason very selective. This can be seen in the chromatograms shown in Figs. 3 and 4. Only the peaks of the target analytes and of the internal standard are detectable. Furthermore on Fig. 4, no interfer-

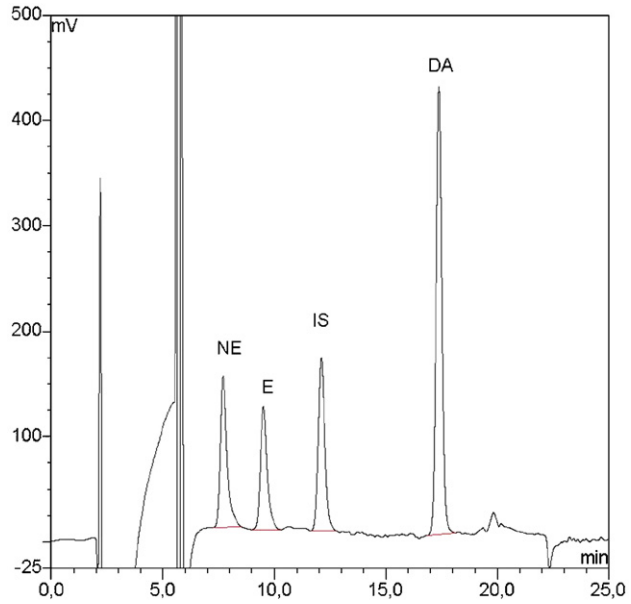


Fig. 3. Representative chromatogram obtained after on-line SPE-LC-ECD analysis of 20 μl of spiked (50 μg/l each of NE, E, DA and 100 μg/l IS) 24 h human urine.

ence peak is observed at the retention time of the internal standard.

Due to the highly efficient and selective clean-up the lifetime of the SPE-column exceeds more than 2000 injections of raw urine samples each of 20 μl. In order to reduce the consumption of the mobile phase used for the extraction step an additional three-port valve was placed between the outlet of the SPE-column and the waste container. Indeed this valve allows the recycling of the extraction mobile phase during the reconditioning step of the SPE-column.

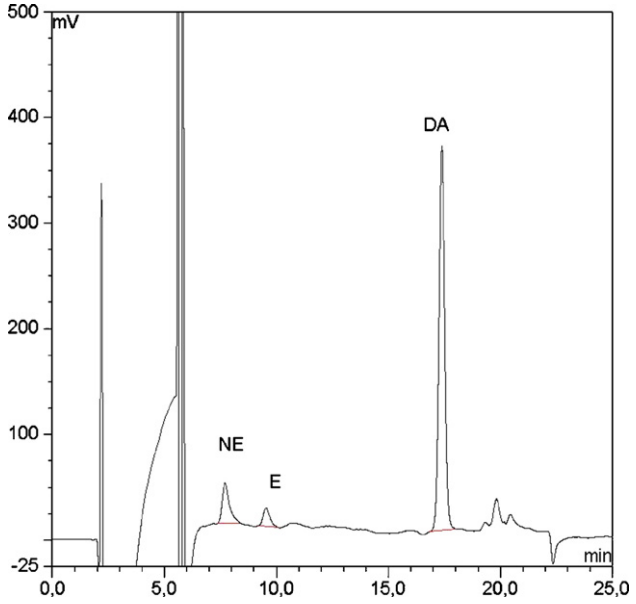


Fig. 4. Representative chromatogram obtained after on-line SPE-LC-ECD analysis of 20 μl of 24 h human urine of a healthy volunteer. NE: 20.9 μg/l; E: 8.7 μg/l; DA: 217.7 μg/l.

An essential prerequisite for a valid bioanalytical method is that the concentration and chemical entity of a given target analyte in a biofluid remain unchanged from the time being sampled until the final analysis. For many analytes the preanalytical phase, i.e. the time between the sample collection and quantitation determines the quality of the overall analytical process [31]. This holds especially for instable analytes such as catecholamines which are easily oxidized. Many of the published protocols fail and lead to false negative results and thus to wrong diagnosis in a clinical setting. Up to now no standardized procedure has been developed in order to completely stabilize these biogenic amines during the overall process.

Recently, we developed a novel collection-set for diagnostic marker molecules in 24 h urine. UriSet 24 (Sarstedt AG & Co., Nümbrecht, Germany) [30] standardizes and guarantees the stabilization of the catecholamines at ambient temperature with 20% hydrochloric acid during the 24 h collection as well as their transport to and storage in the laboratory until analysis.

### 3.2. Method validation

Recently, Nikolajsen and Hansen [32] published a review on analytical methods for determining urinary catecholamines in healthy subjects. They pointed out that many of the reviewed papers lack valuable information and recommended that in future papers the method described should be fully validated. Therefore, an original approach using accuracy profiles based on tolerance intervals for the total measurement error, including both bias and standard deviation for intermediate precision, was applied to demonstrate the method capability [26,27]. The tolerance interval used is a "β-expectation tolerance interval" defining an interval in which it is expected that at least a define proportion of future results (β) will lay inside [29,33,34]. It is, therefore, a predictive tool. This tolerance interval is computed for each validation standard concentration level, using their estimated intermediate precision standard deviation and bias. By joining the upper tolerance limits on one hand and the lower tolerance limits on the other hand, it defines an accuracy profile. As long as this profile stays inside the acceptance limits the method can be considered as valid. Indeed, it guarantees that at least a proportion β (e.g. 0.95 or 95%) of future results will be included in the a priori set acceptance limits.

Such an approach reflects more directly the performance of individual assays and will result in fewer rejected in-study runs than the current procedure that compares point estimates of observed bias and precision with the target acceptance criteria according to the FDA document [35] or Washington conference [36,37]. The concept of accuracy profile was also used to select the most appropriate regression model for calibration, to determine the lower limit of quantitation (LLOQ) and the range over which the method can be considered as valid. The acceptance limits were settled at ±15% according to the regulatory requirements [35–37].

#### 3.2.1. Extraction efficiency

The absolute recoveries of the analytes were determined at three different concentrations ranging from 5 to 500 μg/l

Table 2
Extraction efficiency for norepinephrine, epinephrine and dopamine

| Analyte | Concentration (μg/l) | Analyte recovery (%) ($n=3$) |
|---|---|---|
| Norepinephrine | 15 | 96.3 ± 1.6 |
| | 275 | 95.4 ± 2.1 |
| | 500 | 97.1 ± 1.9 |
| | Mean | 96.3 ± 1.9 |
| Epinephrine | 5 | 95.3 ± 1.8 |
| | 275 | 95.1 ± 1.6 |
| | 500 | 96.6 ± 1.3 |
| | Mean | 95.7 ± 1.6 |
| Dopamine | 50 | 95.6 ± 1.9 |
| | 275 | 96.7 ± 1.7 |
| | 500 | 97.1 ± 2.2 |
| | Mean | 96.5 ± 1.8 |

[35–38]. The mean recoveries are shown in Table 2 for each analyte. Those absolute recoveries were calculated by comparing peak areas for each catecholamine obtained from freshly prepared urine samples treated according to the described procedure with those found after the direct injection on the analytical column of HCl 0.1 M standard solutions at the same concentrations. All the recoveries were high and relatively constant all over the ranges studied, demonstrating the high extraction efficiency of the method.

### 3.2.2. Analysis of the response functions

In order to validate the analytical procedure, the standard addition method was used. Indeed the analytes studied are naturally present in the matrix. Therefore, one calibration curve for each catecholamine was made by spiking 24 h human urine in order to reach 5 concentration levels ranging form 5 to 500 μg/l of added catecholamines. For each calibration curve two calibration standards were analyzed at each concentration level for five days. Independent validation standards at similar concentration levels were also treated in triplicate during the same period. For each time dependent series, the standard addition method was used to obtain the response (signal) of the naturally occurring catecholamines in the urine matrix (i.e. the $y$-axis intercept) as well as the standard error of these values. Those responses were ranging from 8.1 to 9.3 area units (A.U.) for norepinephrine, from 0.7 to 1.9 A.U. for epinephrine and from 56.2 to 77.9 A.U. for dopamine. The maximum standard errors of these responses were of 2.8, 1.0 and 13.0 A.U. for norepinephrine, epinephrine and dopamine, respectively. The values of the $y$-axis intercepts added to their standard errors were subtracted to each analytical response of each analyzed sample in its corresponding series. This step was done for each new series of experiments. The effective calibration curves obtained were made by 4 concentration levels ranging from 15 to 500 μg/l for norepinephrine, by 5 concentration levels ranging from 5 to 500 μg/l for epinephrine and by 3 concentration levels ranging from 50 to 500 μg/l for dopamine.

Then, from these data several response functions were fitted. From every response function obtained for each catecholamine, the concentrations of the validation standards were back-calculated in order to determine, by concentration level, the mean relative bias as well as the upper and lower β-expectation tolerance limits at 95% level by introducing the estimation of the standard deviation for intermediate precision.

From these data, different accuracy profiles were plotted to select the most suitable regression model for the intended use of the analytical method [26–28,38]. As shown in Fig. 5, two response functions, namely the simple linear regression model and the linear regression model after square root transformation, were tested for each catecholamine. The acceptance limits were set at ±15% according to the regulatory requirements [35–37]. Only the linear regression after square root transformation allowed demonstrating the capability of the method to quantify NE and E over the concentration range considered, since the tolerance intervals were totally included inside the acceptance limits. For DA the tolerance intervals for both models were included inside the acceptance limits. Therefore, the simple linear regression was selected since it is an easier model to use. The responses functions obtained by applying these regression models are presented in Table 3.

### 3.2.3. Trueness

Trueness [26,39] expressed in terms of relative bias (%) was assessed from the validation standards for NE, E and DA at 4, 5 and 3 concentration levels, respectively as can be seen in Table 3. According to the regulatory requirements [35], trueness was acceptable for the three analyzed catecholamines, since the bias did not exceed the value of ±15%, irrespective of the concentration level.

### 3.2.4. Precision

The precision of the bioanalytical method was then determined by computing the relative standard deviations (RSD) for repeatability and time-different intermediate precision at each concentration level of the validation standards for the different catecholamines [26,31,32,35,36]. The RSD values presented in Table 3 were relatively low, about 2.4% and 1.9% for NE and E at the lowest concentration levels. However, the maximal RSD value for DA (4.3%) was a little bit higher, but remained still acceptable. These results illustrate the good precision of the developed method.

### 3.2.5. Accuracy, LOQ and LOD

Accuracy takes into account the total error, i.e. the sum of systematic and random errors, related to the test result [26–28,35–39]. As shown in Table 3, the upper and lower β-expectation tolerance limits of the mean bias (%) did not exceed the acceptance limits settled at 15% for each concentration level. Consequently, the method can be considered as accurate over the concentration range investigated [27,35–37]. For NE, E and DA, the lower limits of quantitation (LLOQ) were 15, 5 and 50 μg/l, respectively. As for the limit of detection (LOD), it was estimated using the mean intercept of the calibration model and the residual variance of the regression. The LOD were evaluated at 0.52, 0.26 and 23.98 μg/l, for NE, E and DA, respectively.

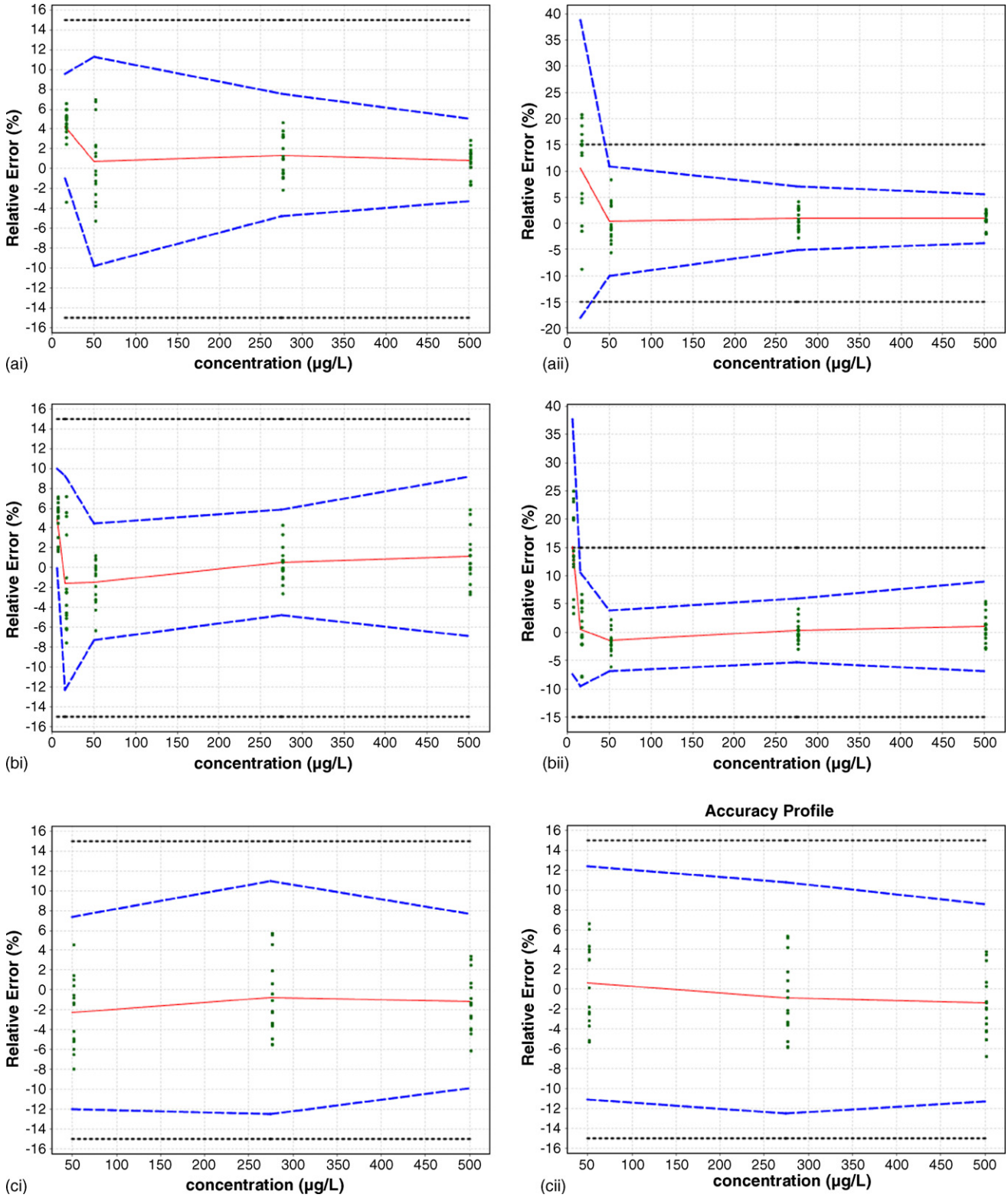


Fig. 5. Accuracy profiles for NE (a), E (b) and DA (c) using a linear regression model after square root transformation (i) or a simple linear regression model (ii). Relative bias (–·–·–), acceptance limits (· · ·), β-expectation tolerance limits (– – –), relative back-calculated concentrations (·).

### 3.2.6. Linearity of the results

In order to demonstrate method linearity [26], a regression line was fitted on the calculated concentrations of the validation standards as a function of the introduced concentrations by applying a linear regression model. The equations obtained for each catecholamine with their coefficient of determination are presented in Table 3.

The slopes values obtained for the three catecholamines were between 0.85 and 1.15, the method can therefore be considered as linear [40]. The linearity of the method was also demonstrated

Table 3
Method validation

| Validation criterion | Norepinephrine | Epinephrine | Dopamine |
|---|---|---|---|
| Response function ($k=5$; $n=2$) | Linear regression after square root transformation<br>Calibration range ($m=4$): 15–500 μg/l | Linear regression after square root transformation<br>Calibration range ($m=5$): 5–500 μg/l | Simple Linear regression<br>Calibration range ($m=3$): 50–500 μg/l |
| Trueness ($k=5$; $n=3$) | | | |
| Relative bias (%) | | | |
| 5 μg/l | – | 4.9 | – |
| 15 μg/l | 4.3 | −1.5 | – |
| 50 μg/l | 0.7 | −1.4 | 0.6 |
| 250 μg/l | 1.4 | 0.5 | −0.9 |
| 500 μg/l | 0.9 | 1.2 | −1.3 |
| Precision ($k=5$; $n=3$) | | | |
| Repeatability/intermediate precision (RSD%) | | | |
| 5 μg/l | – | 1.1/1.9 | – |
| 15 μg/l | 2.3/2.4 | 4.4/4.8 | – |
| 50 μg/l | 2.2/4.0 | 1.5/2.3 | 2.1/4.3 |
| 250 μg/l | 0.9/2.2 | 0.9/1.9 | 1.4/4.1 |
| 500 μg/l | 0.5/1.5 | 0.9/2.8 | 0.9/3.4 |
| Accuracy ($k=5$; $n=3$) | | | |
| β-Expectation lower and upper tolerance limits of the relative error (%) | | | |
| 5 μg/l | – | [−0.1, 10.0] | – |
| 15 μg/l | [−1.0, 9.5] | [−12.3, 9.2] | – |
| 50 μg/l | [−9.8, 11.2] | [−7.3, 4.4] | [−11.1, 12.3] |
| 250 μg/l | [−4.8, 7.5] | [−4.8, 5.8] | [−12.5, 10.7] |
| 500 μg/l | [−3.3, 5.0] | [−6.8, 9.2] | [−11.2, 8.6] |
| Linearity ($k=5$; $n=3$) | | | |
| Range (μg/l) | [15, 500] | [5, 500] | [50, 500] |
| Slope | 1.009 | 1.012 | 0.9846 |
| Intercrept | 0.4496 | −0.6579 | 1.311 |
| $r^2$ | 0.9995 | 0.999 | 0.9965 |
| LOD (μg/l) | 0.5 | 0.26 | 24 |
| LOQ (μg/l) | 15 | 5 | 50 |

using the β-expectation tolerance interval approach. Indeed, as illustrated in Fig. 6a–c, the upper and lower β-expectation tolerance limits were included inside the absolute acceptance limits irrespective to the concentration levels for the three analytes studied.

### 3.3. Risk assessment

By using the β-expectation tolerance intervals obtained with the previously selected regression models, risk of having future measurement falling outside the specified acceptance limits was assessed. This risk is computed as the sum of the proportion of results effectively lying outside the upper acceptance limit on one hand and under the lower acceptance limit on the other hand [41]. The maximum risk tolerated was set at 5%. In other words, this means that it is expected that, in routine analysis, at most 5 sample measurements out of 100 will fall outside the ±15% acceptance limits. Fig. 7 shows the risk profiles for each studied catecholamine constructed by concentration level from the validation standards. In all cases the risk did not exceed 5%. Indeed, the highest risk levels were 1.9% for NE, 2.4% for E and 3.5% for DA. Using these risk profiles the analyst can see how far the analytical method is reliable for its intended use, therefore giving him a new tool to evaluate the reliability of its method. The consumer risk linked to the use of the analytical procedure is known and managed as required in the Process Analytical Technology initiative of the FDA [42].

### 3.4. Uncertainty assessment

In order to interpret correctly results of an analytical procedure, their reliability must be demonstrated. Validation is a first step to achieve this, but is not enough if one aims at interpreting and comparing results correctly. Uncertainty of measurements must therefore be evaluated to ensure this. One major advantage of the applied validation methodology is that it can, without any additional experiments, give estimation of uncertainty of measurements. Indeed Feinberg et al. [43] demonstrated the mathematical link between the variance used to compute the β-expectation tolerance interval and the uncertainty of the measurements. Therefore, as long as the experimental design used for the validation is representative of the sources of variability that will be encountered during routine analysis, this uncertainty estimate is relevant for the results obtained in the laboratory

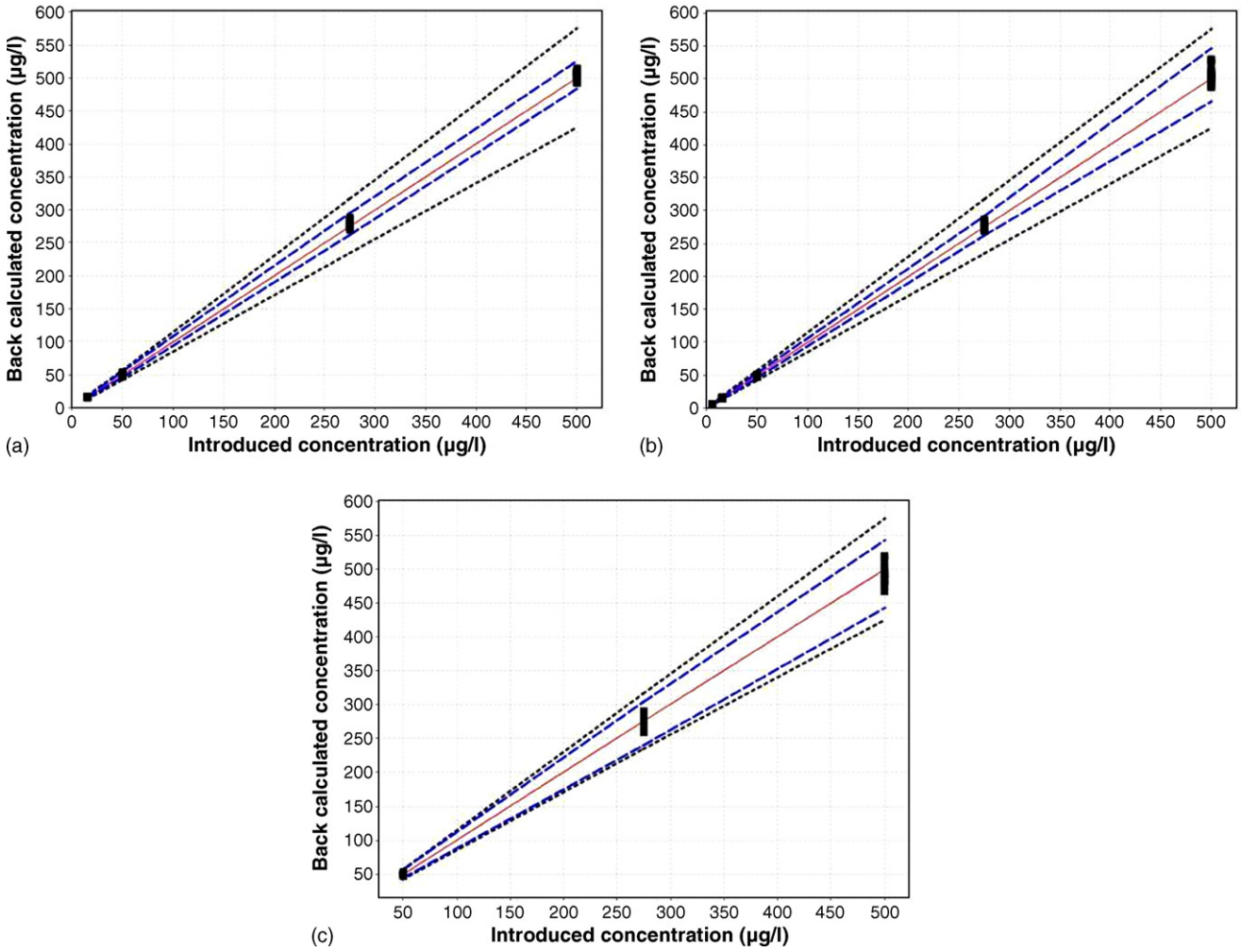


Fig. 6. Linearity profiles for (a) norepinephrine; (b) epinephrine; and (c) dopamine. The continuous line is the identity line (Y = X), the dotted lines are the upper and lower acceptance limits in absolute values and the dashed lines are the upper and lower β-expectation tolerance limits.

having validated the analytical procedure. On this basis, several estimations of uncertainty were computed and are presented in Table 4. The expanded uncertainty was computed using a coverage factor of $k = 2$ [44–46], representing an interval around the results where the unknown true value can be observed with a confidence level of 95%. As shown in Table 4, the relative expanded uncertainty of each catecholamine irrespective of the concentration levels did not exceed 10%. In other words and as long as it was demonstrated in validation that the method is unbiased, this means that with a confidence level of 95% the unknown true value is situated at maximum ±10% around the measured result.

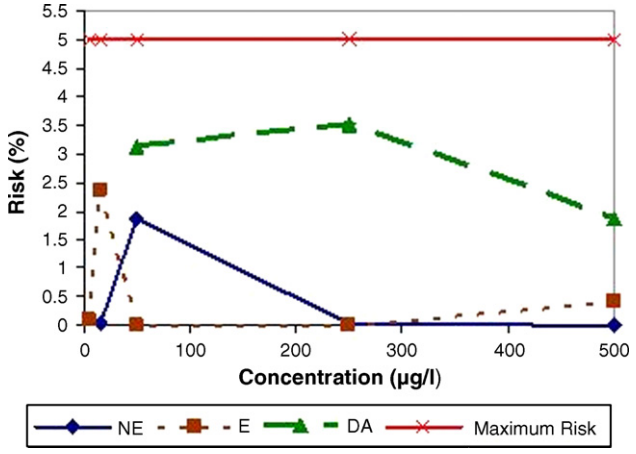


Fig. 7. Risk profiles for NE (continuous line), E (dotted line) and DA (dashed line) obtained by concentration level. The maximum tolerated risk is set at 5%.

### 3.5. Application to real samples and selectivity

To verify the reliability of the on-line SPE-LC-ECD method, samples of 24 h human urine from 21 voluntary donors were analyzed in duplicates. The same samples were also analyzed with the off-line LC-ECD method used in routine analysis at the university hospital of Grosshadern (Munich, Germany). Indeed, as this analytical method is the one currently used to determine the catecholamines concentration of patients of the hospital, it was considered as a gold standard. The sample preparation is handled off-line with a solid phase extraction column using a weak cation exchanger (Chromsystems Instruments, Munich, Germany). Furthermore, due to the complexity of the matrix and

Table 4
Estimates of the measurement uncertainties related to norepinephrine, epinephrine and dopamine, at each concentration level investigated in validation using the selected regression models

| Analyte | Concentration (μg/l) | Uncertainty of the bias (μg/l) | Uncertainty (μg/l) | Expanded uncertainty (μg/l) | Relative expanded uncertainty (%) |
|---|---|---|---|---|---|
| Norepinephrine | 15 | 0.10 | 0.37 | 0.73 | 4.9 |
| | 50 | 0.81 | 2.16 | 4.33 | 8.7 |
| | 275 | 2.57 | 6.59 | 13.18 | 4.8 |
| | 500 | 3.14 | 8.02 | 16.05 | 3.2 |
| Epinephrine | 5 | 0.04 | 0.10 | 0.21 | 4.2 |
| | 15 | 0.21 | 0.75 | 1.49 | 10.0 |
| | 50 | 0.44 | 1.24 | 2.48 | 5.0 |
| | 275 | 2.22 | 5.77 | 11.53 | 4.2 |
| | 500 | 6.01 | 15.16 | 30.33 | 6.1 |
| Dopamine | 50 | 0.89 | 2.35 | 4.70 | 9.4 |
| | 275 | 4.82 | 12.20 | 24.41 | 8.9 |
| | 500 | 7.42 | 18.61 | 37.22 | 7.4 |

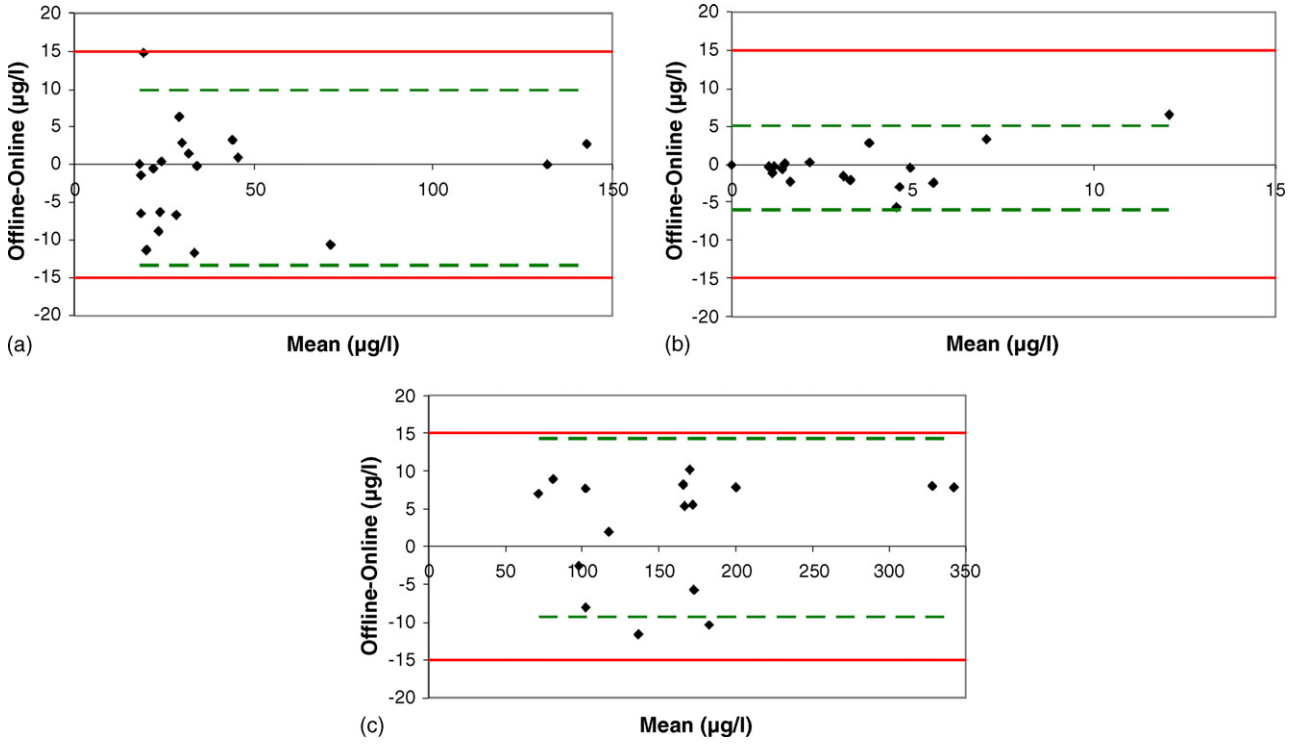


Fig. 8. Differences plots of the mean differences between off-line and on-line LC-ECD methods for (a) norepinephrine; (b) epinephrine; and (c) dopamine. The continuous lines are the ±15% acceptance limits, the dashed lines are the 95% tolerance limits (mean difference ± 1.96 standard deviation).

as no blank matrix is available, this method comparison was also used to assess the selectivity of the on-line method by comparing the results obtained [47] and providing 21 different independent sources of the studied matrix. Therefore, difference plots [48] were constructed as shown in Fig. 8a–c, for norepinephrine, epinephrine and dopamine, respectively. A negative bias was observed for NE and E, whereas a positive bias was observed for DA. However, the two methods can be considered as equivalent, demonstrating the selectivity of the on-line method. Indeed from Fig. 8a–c, it can be seen that the differences of the observed means and the 95% tolerance limits (mean difference ± 1.96 standard deviation) [49] are included inside the acceptance limits of 15% settled according to the Washington conferences [36,37] and the FDA document [35].

## 4. Conclusions

The described fully automated on-line SPE-LC-ECD method is multidimensional and allows the repetitive injection of raw human urine, chemoselective SPE-based sample clean-up, ion-pair reversed phase separation and electrochemical detection of the catecholamines norepinephrine, epinephrine and dopamine.

The fully validated method covers a broad concentration range from physiological to pathological levels. An original validation approach using accuracy profiles based on β-expectation tolerance intervals for the total measurement error permit to indicate the capability of the method. The concept of accuracy profile was also used to select the most appropriate regression model for calibration, to determine the lower limit of quantitation (LLOQ) and the range over which the method can be considered as valid.

Furthermore, the risk with respect to the future use of the validated method was estimated and it was demonstrated that it was less than 5%. The described method is therefore reliable for its intended use, the quantitation of three major catecholamines in human urine being accurate. In addition the measurements uncertainties were estimated without any additional experiments thanks to the validation methodology, allowing correct interpretation and comparison of the results in a cost effective procedure.

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