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An Improved HPLC Method for Quantification of Metanephrine with Coulometric Detection

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Abstract

A rapid and straightforward analytical method, based on the use of RP-HPLC with coulometric detection, was developed and validated for the quantification of metanephrine, an O-methylated product in catechol-O-methyltransferase enzymatic assays. The isocratic separation was achieved on a reverse column with a mobile phase consisting of 0.1 M sodium dihydrogen phosphate, 0.024 M citric acid monohydrate, 0.5 mM sodium octyl sulphate and 9% acetonitrile (%v/v). The method was found to be linear between 0.25 and 15 nmol/mL with a determination coefficient of 0.9997 for metanephrine. Intra-and interday precision and accuracy were in conformity with the criteria accepted in bioanalytical method validation and the LOD and LLOQ were 0.25 nmol/mL. The main focus of the developed method is the lower LLOQ achieved that can have important implications in laboratory research for COMT activity determinations, in particular for the methionine 108/158 variant obtained either from native or recombinant extracts. Another major advantage of the present method is the shorter run times on automated chromatographic systems that allow the analysis of several samples in a short time.

In addition, metanephrine was stable in the samples for at least 24 h at room temperature, for at least 24 h in HPLC system injector and for at least three freeze/thaw cycles. The developed method demonstrated higher sensitivity, precision, accuracy, stability, and linearity when compared with the methods previously described. Finally, a catechol-O-methyltransferase activity assay, resulting in an O-methylated reaction product, was used to evaluate the method applicability.

Keywords: HPLC; Coulometric detection; Metanephrine; Catechol-O-methyltransferase.

Introduction

Normetanephrine (NMN) and metanephrine (MN), O-methylated metabolites of norepinephrine and epinephrine respectively, are produced by the actions of catechol-O-methyltransferase (COMT) (EC 2.1.1.6), an enzyme largely confined to extraneuronal tissues [1], that needs accurate and selective measurements, not only for clinical diagnosis but also for pathological studies of several diseases. In fact, as the catechol-metabolizing system that comprises COMT has a potential pathophysiological and pathogenic significance in several disorders [2,3], it becomes important to the determination of COMT biological activity and determination of metanephrine with lower detection limits and improved sensitivity.

MN quantification, after chemical or enzymatic hydrolysis of the conjugated forms, is still important for diagnosing neural crest tumors, particularly in differentiating between pheochromocytoma and hypertension [4]. Several analytical methods, in particular, HPLC, have been proposed for the analysis of catecholamines and their metabolites (O-methylated reaction products) in biological fluids. Several detecting techniques are depicted in bibliography, namely MS [5], UV spectrophotometry [6], Fluorometry [7] , RIA [8] and chemical luminescence [9].

Liquid chromatography coupled with electrochemical detection (LC-ECD) has provided a new tool to evaluate the levels of these compounds in urine [10], plasma [11] and COMT assays [12] and is considered a reliable technique for catecholamine assay [13]. Typically, catecholamines and their metabolites can be separated by RP HPLC systems with ion-pairing reagents or by ion-exchange HPLC and detected by their reversible oxidation by amperometric [11,12] or coulometric carbon-based working electrodes [14,15]. Analysis of electroactive compounds, like catecholamines, in biological samples/extracts by HPLC with electrochemical detectors in coulometric mode has gained more interest in the last years. As a matter of fact, ECD has been improved since the appearance of the flow-through electrodes (coulometric detectors) that can react with near of 100% of the electroactive components of the analyte. Recently, developed coulometric sensors provide selectivity, identification, and resolution of compounds when used as detectors in HPLC systems. Coulometric detectors claim a better performance for sensitivity and selectivity than the classic amperometric detector, making the coulometric detection an advanced tool capable of addressing the analytical complexity of biological samples/extracts. In fact, coulometric detection in HPLC ECD, a technique in which all of the analyte in the column effluent is oxidized or reduced at the surface of an electrode at constant potential, offer certain advantages over amperometric detection, in which only a few percent of the analyteis converted [16]. A major concernin amperometric HPLC ECD is the decrease of electrode response in time, which is often attributed to the reduction of the active area by adsorption phenomena. On the other hand, when the electrode area of a coulometric detector is large enough, the conversion efficiency may still be 100% despite some loss of active area, so that the response is unaffected [16]. Coulometric detection has been used in detection of

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clinical important substances in Plasma [17], Saliva [18], Urine [19], including catecholamine detection [20].

The sensitivity limitations imposed by the large areas of coulometric (>99% efficient) detectors and the theoretical advantages of 100% detection compared to the 2-5% detection achieved in amperometric sensors, have been recognized with detection limits inside the femtogram (10-15) region [15]. In coulometric detection, 100% oxidation efficiency at one electrode of the analyte and their reduction at a second electrode allow a great increase in selectivity and sensitivity for compounds with reversible oxidation over non-reversible compounds, such as MN, a typical O-methylated product of COMT in the presence of epinephrine. Furthermore, COMT has been described as an important drug target in Parkinson's disease [2], emphasizing the importance of the development of methods with improved LLOQ that allow the quantification of lower MN levels and, consequently, the determination of low enzyme activity levels.

In this work, we describe a novel method using HPLC with coulometric detection, with adequate sensitivity for reliable measurement of MN in biological bacteria lysate extracts for the first time, by calibration procedures, including intra- and interday precision and stability. Moreover, this method was fully validated in a wide concentration range with lower limit of quantification. We evaluated the method applicability through in vitro membrane COMT activity assays, using epinephrine as substrate, resulting in an O-methylated reaction product such as MN. The method reported here can be applied to clinical MN assessment on biological fluids (plasma, urine) after suitable extraction procedures due to the high sensitivity and speed shown, associated to a greater selectivity of the coulometric detectors.

Experimental

Reagents and standards

Analytical standards of MN (DL-Metanephrine Hydrochloride), citric acid monohydrate and 1-Octanesulfonic acid were purchased from Sigma Aldrich (Steinheim, Germany). Acetonitrile (HPLC grade) and sodium dihydrogen phosphate were obtained from Fisher Scientific (Leicestershire, UK) and USB Corporation (Ohio, USA), respectively. Perchloric acid was purchased from Panreac (Barcelona, Spain). Deionized water was obtained from a Millipore purification system in our research laboratory. Stock solution at 25 nmol/mL of MN was prepared in 0.2 M perchloric acid. Standard solutions were obtained by diluting stock solution with the same solution of perchloric acid. All these buffers were stored and protected from light at 4°C and were stable for at least three months.

Instrumentation

Chromatographic analysis was performed using a HPLC model Agilent 1260 system (Agilent, Santa Clara, California, USA) equipped with an autosampler and quaternary pump coupled to an ESA Coulochem III (Milford, Massachussets, USA) coulometric detector. Chromatographic separation was achieved on an analytical column Zorbax 300SB C_{18} RP analytical column (250×4.6 mm i.d. 5 µm) (Agilent, Santa Clara, California, USA). The mobile phase (0.1 M sodium dihydrogen phosphate, 0.024 M citric acid monohydrate, 0.5 mM sodium octyl sulphate and 9% acetonitrile, v/v), pH 2.9, was filtered under vacuum (0.2 µM hydrophilic polypropylene filter), degassed in ultrasonic bath before use. Under this procedure the mobile phase was maintained stable during 2 weeks. Column effluent was monitored with an electrochemical detector in the coulometric mode, which was equipped with a 5011 high sensitivity dual electrode analytical cell (electrodes I and II) using a procedure of oxidation/reduction (analytical cell #1: +410 mV; analytical cell #2: -350 mV). The high surface area of electrodes I and II results in a 100% reaction of the electroactive compound (MN). The method sensitivity was set at 1 µA and the flow rate applied was 1mL/min. Column temperature was optimized to 30°C. The chromatograms were obtained by monitoring the reduction signal of the working electrode II. The retention time was around 8.8 min for MN.

Standards preparation

A stock solution of MN (25 nmol/mL) was prepared by dissolving the appropriate salt in 0.2 M perchloric acid. The initial solution was diluted in 0.2 M perchloric acid in order to obtain MN standard solutions at several concentrations (0.25 to 15 nmol/mL). The standard samples were agitated by rotation/inversion movements for 2 min and injected into the HPLC-ECD system according to the experimental conditions.

Validation procedure

The procedure was validated in terms of selectivity, linearity, intra- and inter-day precision, accuracy, and stability. Calibration data was generated by spiking samples and the calibration curve was established between 0.25 and 15 nmol/mL (eight calibrators evenly distributed). Five calibration curves were prepared, and the criteria for acceptance included a R^2 value of at least 0.99, and the calibrators accuracy within a ± 15% interval, except at the lower LLOQ (LLOQ), for which ± 20% was accepted. The LLOQ was defined as the lowest amount of analyte that presented a signal-to-noise ratio of at least 5 and could be measured with adequate precision and accuracy (coefficient of variation of less than 20% and an accuracy of ± 20%). Intraday precision was characterized in terms of RSD (%) by analyzing sets of 5 MN samples at five different concentrations (0.25, 2, 4, 8 and 12 nmol/mL) over a 5-day period. Interday precision was assessed at eight concentrations (0.25, 0.5, 1, 2, 4, 8, 12, 15 nmol/mL) over a 5-day period. Accuracy was evaluated in terms of mean relative error between the measured and the spiked concentrations for the calibrators and also in the intra- and interday precision assays; the limits of acceptable variability were set at 15% for all concentrations, except at the LLOQ, for which 20% was accepted. Processed sample-stability, short-term stability and freeze/thaw stability were studied (n=3) at two concentration levels (3 and 10 nmol/mL).

MN samples were subjected to different storage conditions, and the obtained results were compared to those achieved after analysis of freshly prepared samples. The compound was considered stable under the tested conditions if the coefficients of variation between the two sets of samples were less than 15%.

Membrane-bound catechol-O-methyltransferase expression and enzymatic assay

Recombinant human membrane-bound catechol-O-methyltransferase (hMBCOMT) biosynthesis was carried out according to the procedure previously described by Pedro et al. [3]. Cells were grown in a mineral salt medium supplemented with soytone 1% (w/v) for 50 hrs at 30°C and 120 rpm. The experiments of activity were designed to evaluate the methylation efficiency of recombinant hMBCOMT, by measuring the amount of MN, using epinephrine as substrate, as previously described by Passarinha et al. [12]. In hMBCOMT activity assay, a 2000 µg/mL aliquot of the membrane extract after suitable solubilization, was incubated in 5 mM sodium phosphate buffer (pH 7.8) containing 0.2 µM $MgCl_2$, 2 mM EGTA, 250

µM SAM e and 1 mM epinephrine in a total sample volume of 1 mL. Reactions were carried out at 37°C during 15 min and were stopped by incubation in ice following the addition of 2M 200 µl perchloric acid. The supernatants were centrifuged, filtered and subsequently injected into the HPLC system.

Results and Discussion

The methodology presented in this work concerns the development and validation of an HPLC method with coulometric detection for assessment of MN, allowing the determination of the biological activity of recombinant COMT as well as native COMT extracted from animal tissues or cell lines. This method was validated using authentic samples obtained from a bioprocess intended to synthesize the membrane-bound isoform of COMT [3]. On the other hand, in humans, the activity of COMT can be distributed into three classes with high, intermediate and low activity groups in which such difference is correlated with a functional polymorphism at codon 108/158 (SCOMT/MBCOMT) involving a methionine/valine substitution in the polypeptide chain [2]. While the metionine 108/158 variant is associated with low enzymatic activity [2], the development of HPLC methods with improved LLOQ and sensitivity capable of measuring lower quantities of MN seems to be of great importance not only to monitor bioprocesses by measuring the biological activity of the target recombinant COMT but also to measure the biological activity of low activity native COMT extracted from animal tissues or cell lines in different pathophysiological and pathogenic conditions.

Chromatographic methodology

The chromatographic conditions were chosen to allow selectivity on the basis of the ionic and hydrophobic characteristics of MN. Chromatographic parameters such as temperature and mobile phase (data not shown) were investigated in order to obtain a higher detection of MN within an acceptable time span. Cell potentials were optimized according to preliminary experiments (data not shown) and ESA Biosciences, Inc indications. The C18 stationary phase has been successfully employed for the separation and quantification of catecholamines and its metabolites, as previously shown by Passarinha et al. [12], Hollenbach and collaborators [21] and Unceta [22]. Therefore and based on these results, the C_{18} was applied as the stationary phase in this work.

In order to obtain better electrochemical oxidation efficiency, the larger electrode surface area coulometric cell, compared to the amperometric, results in a 99% reaction of our electroactive compound-MN. While the electrochemical behavior of MN is reversible at carbon electrodes, we used the oxidation/reduction mode in the coulometric detector because it plays an important role in improving both selectivity and sensitivity of our analysis. The electrode 1 (E1-oxidation channel) was applied only to modify the molecules and reduce contaminants in the sample before the detection on electrode 2 (E2-reduction channel). In the E1, MN was oxidized generating oxidation products which were reduced at E2. In this way, monitoring of the channel E2 current eliminates a great deal of the interferences and improves the signal/noise ratio, emphasizing the chromatographic response for MN (see Figures 1 and 2).

Method Validation

The method was validated in a 5-day validation protocol. The validation parameters included linearity and LLOQ, intra-and interday precision and accuracy, and stability were performed according to the

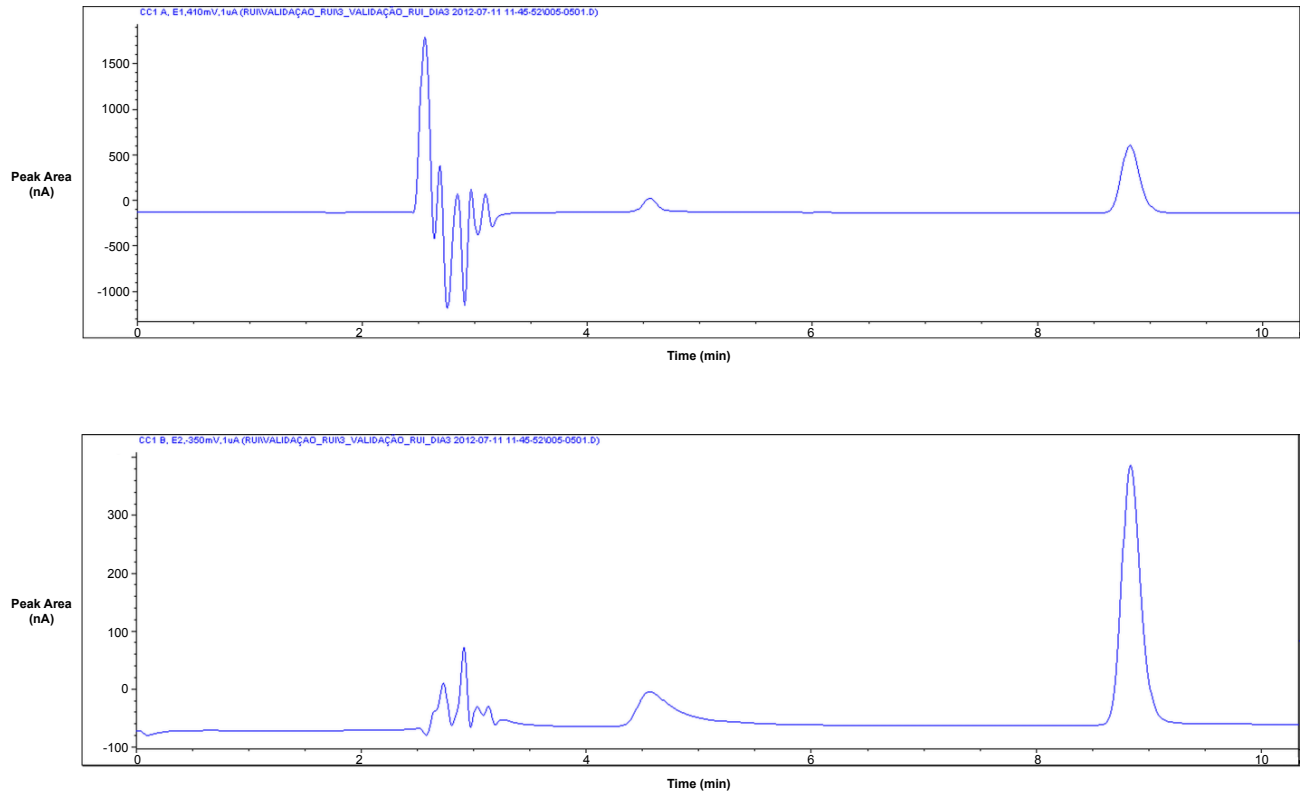

Figure 1: Chromatogram of MN at 2 nmol*mL^{-1}; (a) E1-Oxidation, (b) E2-Reduction (down); retention time (MN) of 8.873 min.

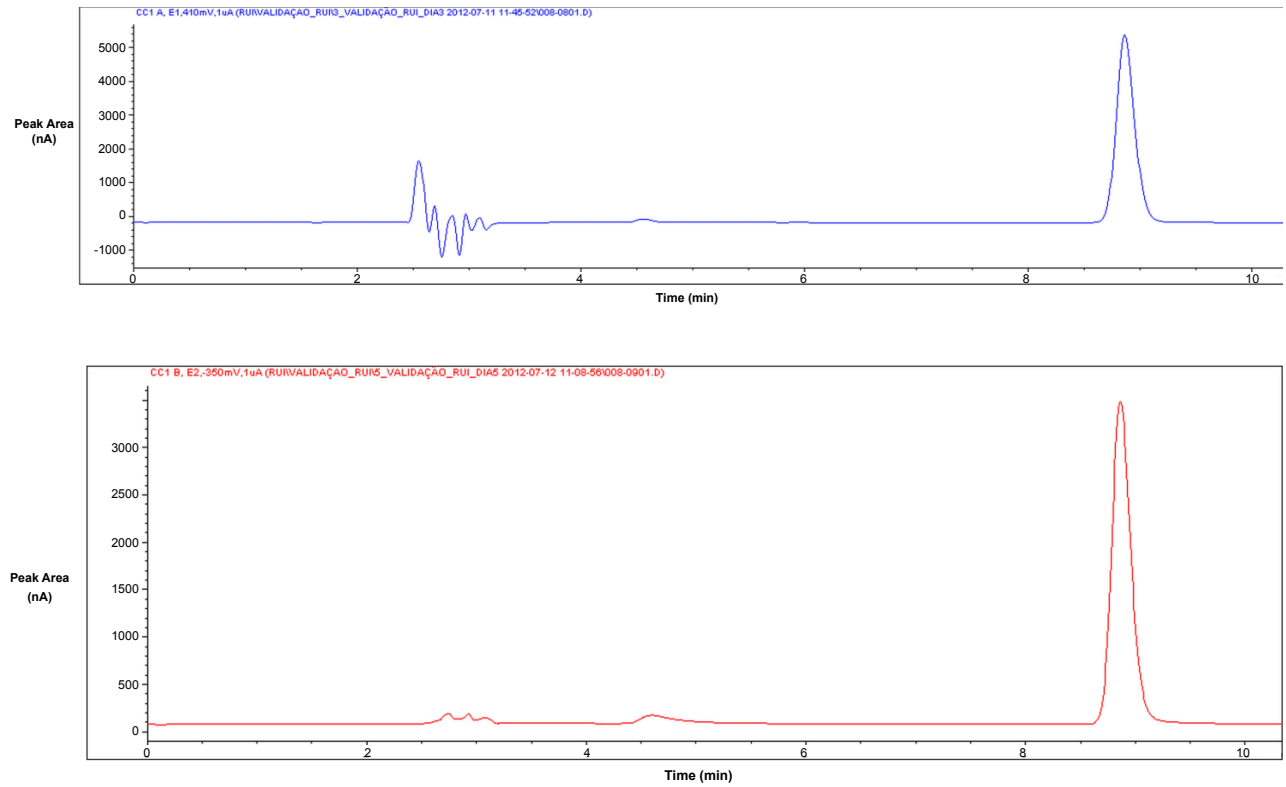

Figure 2: Chromatogram of MN at 15 nmol*mL^{-1}; (a) E1-Oxidation, (b) E2-Reduction (down); retention time (MN) of 8.873 min.

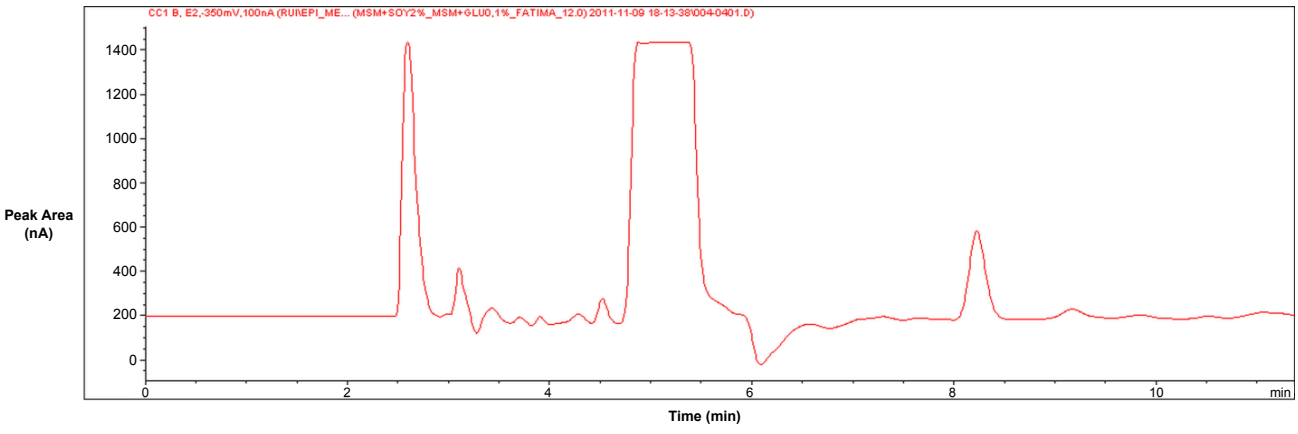

Figure 3: Chromatogram of a biological extract sample (Retention time (MN) of 8.220 min).

guidelines principles of the Food and Drug Administration [23] and International Conference on Harmonization [24].

As shown in Figure 4, the HPLC chromatogram of a fermentation extract sample demonstrates that the method provides an excellent resolution and selectivity between the compounds of interest, allowing the samples to be directly injected without pretreatment. In addition, the different components of the bacteria extract sample were injected as a "blank analysis" in order to evaluate possible interferents at the respective retention times of the analyte but none was observed.

The linearity of the method for MN quantification was established between 0.25 and 15 nmol/mL with eight evenly distributed calibrators and the following values were obtained (mean values ± SD): 2943.4 ± 24.2664 for slope (m), 126.3828 ± 76.0743 for intercept (b) and a R^2 of 0.9997 ± 0.0001. Each calibration level was in quintuplicate and determination coefficients higher than 0.99 were obtained. The calibration curves were obtained by plotting the peak area ratio between the analyte versus analyte concentration. The calculated concentration of each calibrator had to be within a ± 15% interval of target except for the LLOQ, where ± 20% was accepted. In addition, to each calibration curve, two control samples were prepared in triplicate (n=15) at the concentration 3 and 10 n nmol/mL, the low quality control (LQC)

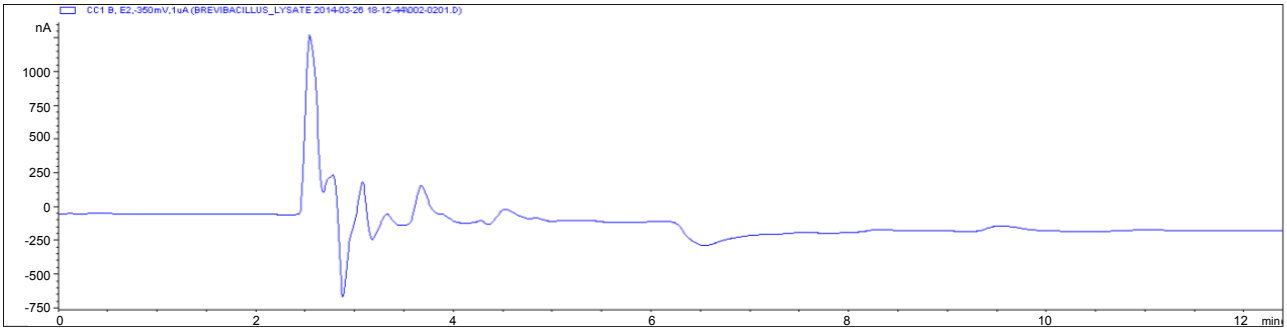

Figure 4: Representative chromatogram of a *Brevibacillus choshinensis* lysate extract sample (blank).

	Measured concentration		CV (%)		RE (%)	
Spiked	**Intraday (n=6)**	**Interday (n=5)**	**Intraday (n=6)**	**Interday (n=5)**	**Intraday (n=6)**	**Interday (n=5)**
0.25	0.28 ± 0.01	0.24 ± 0.02	4.36	8.78	11.55	-4.13
0.5	-	0.51 ± 0.02	-	4.52	-	1.42
1	-	1.01 ± 0.04	-	4.16	-	1.33
2	1.98 ± 0.02	1.98 ± 0.04	1.03	1.96	-0.85	-0.87
4	3.94 ± 0.03	3.94 ± 0.07	0.74	1.67	-1.57	-1.56
8	7.94 ± 0.03	8.10 ± 0.07	0.33	0.87	-0.79	1.22
12	11.81 ± 0.05	12.03 ± 0.14	0.47	1.18	-1.60	0.28
15	-	14.94 ± 0.13	-	0.88	-	-0.40

All concentrations in nmol*mL^{-1}; CV, coefficient of variation; RE, relative error [(measured concentration−spiked concentration)/spiked concentration×100].

Table 1: Intra-day and Inter-day precision and accuracy (n=5).

and high quality control (HQC) respectively. The LLOQ was defined as the smallest concentration of analyte (MN) that could be measured reproducibly and accurately (coefficient of variation less than 20% and calculated concentration within a ±20% interval from the target level) and was established at 0.25 nmol/mL. The LOD, was not systematically evaluated, and was defined to be the same as the LLOQ for practical reasons.

On the other hand, since the coulometric detector presents a high sensitivity, this parameter was set at 1 μA, allowing the detection of the MN standards with higher concentration without channel saturation and not compromising the detection of low standards. Moreover, following this methodology, a retention time around 8.8 min for MN was obtained, faster and more stable hands-on sample than reported by Gamache et al. [25] and Lenders [11] using coulometric detection and better precision/accuracy and lower retention time than reported by Passarinha et al. [12] and Parker and collaborators [26] with amperometric detection. In addition, our method showed a better efficiency and sensitivity, being able to detect MN concentrations of 0.25 nmol/mL with an excellent signal-to-noise ratio, better than reported by amperometric detectors [12], making the coulometric detection an advantageous method to MN quantification.

Other parameters evaluated for this method were the intraday, interday and intermediate precisions. In what concerns to the intraday precision and accuracy (relative error, %) for MN, it was determined by analysis of six independent replicates at five concentrations across the dynamic range of the assay: 0.25; 2; 4; 8 and 12 nmol/mL. The obtained CVs were typically below 5% at all concentrations, while relative errors were within a ±12% interval (see Table 1).

In addition, the interday precision and trueness were evaluated at eight concentrations within a 5-day period. The analysis of the interday precision and trueness yielded CVs generally lower than 9% at all concentration levels, while trueness was within a ±5% interval (see Table 1).

Another parameter evaluated in this work was the intermediate precision (combined intra- and interday) that was determined using the QC samples (LQC and HQC) that were prepared and analysed simultaneously with the calibration curves on 5 different days (15 measurements for each concentration). The CVs were typically below 2% at all concentrations, while accuracy was within ±6% of the nominal concentration.

Finally, the stability of metanephrine was assessed using samples spiked at the above-mentioned QC concentrations (n=3). In order to study the stability of the processed samples, the samples were left standing at room temperature and in the HPLC autosampler for 24 hrs. Those samples were compared to freshly prepared samples, and the obtained coefficients of variation were less than 10% for all compounds, meaning that MN are stable in the samples for at least 24 h at room temperature. Furthermore, the freeze/thaw stability was evaluated as follows: the MN QC samples were spiked at the intended concentrations and stored at −20°C for 1hrs and after this period, the samples were thawed single-handedly at room temperature. Following the third freeze/thaw cycle, the samples were analyzed and the obtained peak areas were compared to those obtained by analysis of freshly prepared samples. The MN was found to be stable for at least 3 freeze/thaw cycles (the obtained CVs were less than 10% for all compounds) with all values lying within ±15% and, hence, were considered acceptable (see Table 2). Also, the target analyte shows stability at room and injector temperature (4°C) up to 24 hrs, which is in accordance with previous findings [27]. Extended storage is possible at -20°C, because at least 3 freeze-thaw cycles had no influence on MN detection.

Method application with biological samples

After validation, the described procedure was applied in three

	Stability at room temperature			Stability of injector			Freeze stability		
Spiked	Measured	CV (%)	RE (%)	Measured	CV (%)	RE (%)	Measured	CV (%)	RE (%)
3	2.63 ± 0.28	9.83	-12.49	2.72 ± 0.13	2.72	-9.45	2.63 ± 0.26	9.81	-12.48
10	9.45 ± 0.93	9.89	-5.53	9.70 ± 0.58	9.70	-3.01	9.51 ± 0.84	8.81	-4.86

All concentrations in nmol*mL^{-1}; CV, coefficient of variation; RE, relative error [(measured concentration-spiked concentration/spiked concentration)×100]

Table 2: Stability data (n=3).

biological samples obtained from a MB-COMT enzymatic activity assay. A typical chromatogram of one of these biological samples is shown in Figure 3. Moreover, as MN is susceptible to oxidation due to the presence of the catechol structure, the presence of a reducing agent in standard solutions (perchloric acid 0.2 M) usually guarantees a higher stability of the analyte avoiding the oxidative degradation products of MN, which strongly influence the accuracy of the analysis [12]. Therefore, as can be seen in the chromatogram of a biological sample (see Figure 3), is possible to observe extra peaks corresponding to perchloric acid and epinephrine (used as substrate in MB-COMT enzymatic assay). However, these peaks don't interfere with MN (reaction product of MB-COMT enzymatic assay) in terms of magnitude and shape of peaks. In addition, all the compounds of the sample, namely MN, give neat and resolved chromatographic peaks as well as a very good selectivity to our interest compound. The mean concentration of MN present in the 3 biological samples was 1.499 ± 0.0135 nmol/mL. Finally, in order to evaluate possible interferents that could overlapping at the respective retention time of MN, a blank (bacteria lysate extract deproteinized with percloric acid) was injected into the HPLC system with the same operatory conditions (Figure 4). As we can observed for the retention time range of 8 to 9 min none components from the bacteria extract sample can be visualized, demonstrating that the analytical method develop is appropriated for this biological matrices.

Conclusions

A simple procedure employing a HPLC system with coulometric detector was developed and fully validated for the qualitative and quantitative determination of MNs from MB-COMT recombinant *Brevibacillu schosinensis* extracts. The proposed HPLC method with ECD, which employs an innovative coulometric detection in MB-COMT enzymatic assays, is suitable for the analysis of the main endogenous catecholamines, namely epinephrine and its main metabolite MN.

The optimization of the chromatographic conditions led to a more rapid, efficient and sensitive method, allowing the clear determination of MNs in MB-COMT enzymatic assays. The HPLC-ECD method developed in this work, in comparison with previously reported methods, namely the metanephrine detection quantification by HPLC coupled with amperometric detection [12], presents shorter chromatographic runs (10 minutes in contrast with 20 minutes). In addition, this method also presents a very good resolution to detect MN and the greater sensitivity associated with the coulometric detector, in contrast to other existing methods. Finally, another major advantage of this method is the improvement on the signal/noise ratio achieved that allowed us to lower the LLOQ to metanephrine from 1 nmol/mL [12] to 0.25 nmol/mL^{-1}, what can be of extremely importance in determining low COMT activity levels.

The method sensitivity, linearity, limits and intra- and interday precision and accuracy were adequate, allowing MN detection even at very low concentrations. Therefore, this procedure may be useful both in research and in routine analysis, more specifically in the quantification of low metanephrine concentrations associated with the COMT biological activity obtained either from recombinant or native sources. In conclusion, the accurate determination of lower metanephrine levels within a short time analysis provided by this method may have important implications in COMT research and, consequently, in the development of new and more effective COMT inhibitors for application in Parkinson's disease.

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