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# A simple and rapid HPLC–MS method for the simultaneous determination of epinephrine, norepinephrine, dopamine and 5-hydroxytryptamine: Application to the secretion of bovine chromaffin cell cultures

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**Abstract**

This method simultaneously determines epinephrine, norepinephrine, dopamine and 5-hydroxytryptamine by HPLC coupled to atmospheric pressure chemical ionization mass spectrometry, using bovine chromaffin cells to test xenobiotic neurotoxicity and the secretion alterations of these neurotransmitters as endpoint. Chromatographic separation was developed by injecting the sample without previous treatment into a reversed-phase column. The signal was recorded in selected ion mode. The lowest limit of detection was found for hydroxytryptamine, while the highest limit was for norepinephrine. The feasibility of the proposed method was checked by performing measurements of neurotransmitters during the assessment the effect of mipafox on the basal and potassium-induced secretions of chromaffin cell cultures.


*Keywords:* Chromaffin cell secretion; Neurotransmitters determination; HPLC–MS; Mipafox; Catecholamine

## 1. Introduction

Adrenomedullary chromaffin cells are embryologically developed from the neural crest [1] and are thus considered as a paraneurons [2]. Indeed, these cells are considered as a good model for the study of Neuropathy Target Esterase, the target of the neurodegenerative process induced by certain organophosphorus compounds [3,4]. In addition, these cells have been widely employed as in vitro model for the study of the mechanisms, pharmacology and biochemistry of neurosecretion. Chromaffin cells have also been employed as in vitro model to test the neurotoxicity of many substances such as vegetal, bacterial and animal biotoxins, heavy metals, organophosphorus compounds, etc. [5].

It is well established that the depolarization of the plasma membrane of chromaffin cells (evoked by either the pulse of $K^+$ or nicotinic channel agonist) causes the opening of the voltage-dependent calcium channels with a concurrent increase of the intracellular calcium concentration. Finally, when calcium reaches a critical concentration, secretion takes places. Secretions of chromaffin cells contain catecholamines and neurotransmitters (NT) such as epinephrine (E), norepinephrine (NE), dopamine (D) and 5-hydroxytryptamine (5-HT); ATP and adenine nucleotides; chromogranins; bioactive peptides such as opioid peptides and enkephalins and neuron specific proteins [4].

The use of animals in toxicological assessments has ethical, social, scientific and even legislative concerns. Nowadays there is, therefore, great pressure concerning the development of the so-called alternative methods, where cell cultures are one of the most employed alternatives. However, it is necessary to characterize a good toxicological endpoint before proposing a cellular system as an in vitro method to test any adverse effects of xenobiotics. According to their physiological function, one of the most appropriate endpoints for chromaffin cells would be the determination of alterations in the secretion pattern. Therefore,

*Abbreviations:* 5-HT, 5-hydroxytryptamine; D, dopamine; DMEM, Dulbecco's modified Eagle's medium; E, epinephrine; K-B-B, Krebs-bicarbonate basal buffer pH 7.4; MS, mass spectrometry; NE, norepinephrine; NT, neurotransmitters

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it would be ideal to ascertain simple, fast and reliable methods for the determination of catecholamines and NT.

The most popular methods to identify and quantify NT in biological fluids are based on GC–MS or HPLC with UV, or on either the electrochemical or fluorescence detector [6]. The main drawback of GC–MS analysis is the absolute necessity of sample derivatization to convert the analyte into a more volatile compound [6]. HPLC-based methods also have several inconveniences since they often require a derivatization to convert NT into a fluorescent molecule [6–8], and/or a previous extraction for either the concentrating or previous purification of the sample [6,9].

Mass spectrometry (MS) coupled to HPLC is a powerful technique with many applications within the field of analytical toxicology [10,11], and it has also been used for the analysis of catecholamine and other NT in several biological fluids [6,12–15]. Nevertheless, most of these methods have several inconveniences, such as the necessity of previously treating the sample or the chromatographic separation by ion pair reagents, which typically hinder MS detection.

This work describes a simple, reliable and fast method for the simultaneous detection of E, NE, D and 5-HT secreted by chromaffin cells. This method features the direct injection of the sample into a HPLC system with a phase-reverse column and further detection by atmospheric pressure chemical ionization mass spectrometry. One main advantage of this method is that the sample requires no treatment prior to the injection. The method displays enough sensibility, precision and accuracy to be used to test alterations in the secretion of these NT by chromaffin cells as an endpoint in the assessment of xenobiotic neurotoxicity.

## 2. Experimental

### 2.1. Chemicals

Mipafox (*N*,*N*′-diisopropyl diamido phosphofluoridate) with a purity higher than 99% was purchased from Lark Enterprises (Webster, MA, USA). All chemicals were supplied by Sigma Quimica España S.A. (Madrid, España) and were either the analytical or HPLC grade.

### 2.2. Cell isolation and culture

Bovine adrenal glands were obtained from Alicante (the local slaughterhouse) within 15–20 min after the animal's death, and were taken to the laboratory in the next 30 min. Chromaffin cells were isolated from these adrenal glands following standard protocols [16,17]. In short, adrenal glands were digested through perfusion with a solution of 0.25% collagenase, 0.01% DNAase and 0.5% bovine serum albumin. Afterwards, the chromaffin cells were purified from debris and erythrocytes using a Percoll gradient developed in a Locke buffer (5.0 mM HEPES/154 mM NaCl/5.6 mM KCl/3.6 mM $NaHCO_3$/5.6 mM glucose pH 7.4). Cells were seeded in a monolayer at 200,000 cells/well in COSTAR trays previously coated with poly-L-lysine. Cultures were maintained at 37 °C under an atmosphere of 95% air and 5% $CO_2$ using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 10 μM cytosine arabinoside, 10 μM 5-fluoro-2′-deoxyuridine, 25 IU penicillin/mL and 25 mg streptomycin/mL. The viability of the cells was assessed using the Trypan blue exclusion test, which was always higher than 95%. All experiments begun on the third day after seeding.

### 2.3. Chromatographic conditions

The HPLC system consisted of an Agilent HP 1100 with two pumps and automatic sampler. A Zorbax (XDB-$C_8$) 5 μm reversed-phase analytical column (150 mm × 4.6 mm i.d.) was used to separate NT. Chromatographic separation was carried out using a gradient (methanol–3 g/L acetic acid, pH 2.8) that increased the methanol concentration of the mixture from 0 to 60% in 6 min with a flow of 0.75 mL/min. The injection volume was 25 μL.

### 2.4. Mass spectrometry detection

The atmospheric pressure chemical ionization MS detector worked in the positive ionization mode. Nebulization was carried out at 300 °C and 50 psi, under a dryer stream of 12 L $N_2$/min. The capillary voltage was set at 3000 V. Secretions were quantitatively determined by recording the abundance of the *m*/*z* fragments 152, 166, 137 and 160, that corresponded to NE, E, D and 5-HT, respectively.

### 2.5. Method validation

Our initial aim was apply the method by direct injecting of the biological samples (without any previous treatment of purification, derivatization or concentration). Thus, in order to gain enough sensitivity and to reduce background, we chose for monitorization only the most abundant *m*/*z* relationships for each of the NT. It yielded with biological samples the baseline displayed in Fig. 3.

Calibrations curves were performed by injecting (three independent experiments) 14 different standard solutions of concentrations between 0.5 and 4000 ng/mL of NE, E and 5-HT and between 0.5 and 1000 ng D/mL. The recorded signals (zero included) were plotted against concentration. Experimental data were analyzed with lineal or exponential models using SIGMA Plot 8.0 software.

Detection and quantification limits were estimated as the lowest concentrations of NT that provided a peak which yielded a signal/noise ratio higher than 3 and 5, respectively. Precision was defined as the coefficient of variation for four independent experiments. Accuracy was calculated as: $100 \times (E - T)/T$, where $E$ is defined as the calculated concentration using the corresponding calibration curve and $T$ is the nominal concentration of the standard sample. The number of plates of each peak were calculated as: $N = 16\,(t_R/W)^2$, where $W$ was the peak width and $t_R$ the retention time. The separation factor ($\propto$) was calculated as the ratio between capacity factors, while resolution ($R$) was calculated using the following equation: $R = 2\,(t_{R2} - t_{R1})/(W_2 - W_1)$.

### 2.6. Cell treatment

Three days after, the culture chromaffin cells were washed 3 times with 100 μL of Krebs-bicarbonate basal buffer pH 7.4 (K-B-B) with the following composition (in mM): NaCl 119; KCl 4.7; $MgSO_4$ 1.2; $CaCl_2$ 2.7; $NaHCO_3$ 10; ascorbic acid 0.6; glucose 11. Afterwards, monolayers were treated 60 min at 37 °C with 200 μL of either K-B-B (control) or 75 μM mipafox.

### 2.7. Neurotransmitter secretions

Immediately after treatment with mipafox, the organophosphorus compound and K-B-B were removed, and cells were washed 3 times with 100 μL of fresh K-B-B. In order to collect the basal secretion of NT, cells were incubated 5 min at 37 °C with 100 μL of K-B-B. After removing the K-B-B, the secretion was induced by incubating monolayers for 5 min at 37 °C with 100 μL of Krebs-bicarbonate 75 mM potassium buffer pH 7.4 (a buffer isoosmotically similar to K-B-B with 75 mM KCl and 63.7 mM NaCl). Secretions were removed and cells were incubated for a further 5 min with 100 μL of K-B-B in order to remove the NT which were non-specifically bound to cellular membranes. The addition of these two last secretions was considered to be the induced secretion. Finally, the intracellular content of NT was determined after lysating cells with 100 μL of 0.4 M $HClO_4$ for 20 min. In each case, all secretions were stored in vials of chromatography at −80 °C until the time of analysis.

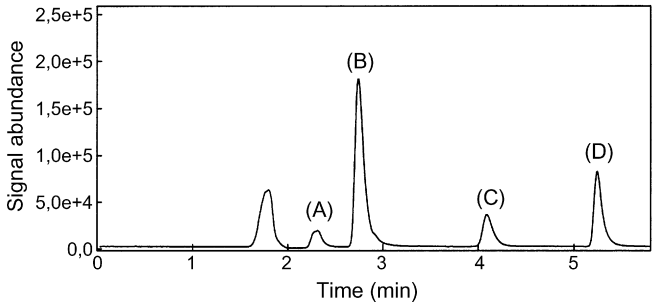


Fig. 1. Chromatogram of a standard mixture of neurotransmitter. The chromatogram was obtained by injecting 25 μL of a standard sample containing 0.125 μg/mL of norepinephrine (peak A), epinephrine (peak B), dopamine (peak C) and 5-hydroxytryptamine (peak D). The separation and detection conditions are described in Section 2. Each peak was identified by comparing the retention times (see Table 1) after the injection of solutions with only one NT and with full scan mass spectra (see Fig. 2).

## 3. Results

### 3.1. Method performance and validation

Fig. 1 displays the full chromatogram of a standard sample (panel A). The peaks of each NT were identified through a com-

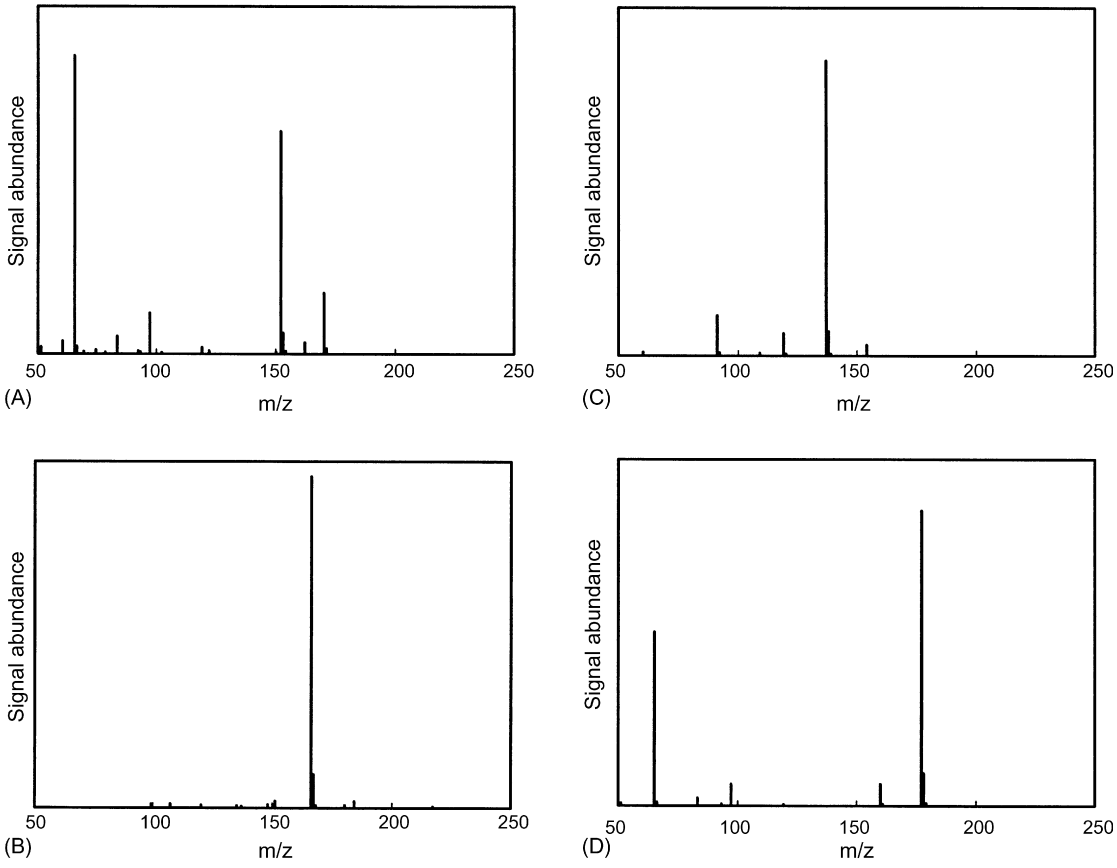


Fig. 2. Full scan mass spectra of norepinephrine (panel A), epinephrine (panel B), dopamine (panel C) and 5-hydroxytryptamine (panel D). Mass spectra were obtained through the chromatogram displayed in Fig. 1. The ionization conditions are described in Section 2.

Table 1
Chromatographic parameters in the method

| Neurotransmitter | $t_R$ (min) | $N$ | $\propto$ | $R$ |
|---|---|---|---|---|
| NE | 2.29 | 7,330 | – | – |
| E | 2.76 | 10,800 | 1.9 | 4.4 |
| D | 4.17 | 21,800 | 2.4 | 13 |
| 5-HT | 5.27 | 61,200 | 1.5 | 11 |

Retention time ($t_R$), number of plates ($N$), separation factor ($\propto$) and resolution ($R$) were calculated using the data from the chromatogram displayed in Fig. 1.

parison of retention times (Table 1) and with the full scan spectra (Fig. 2) of runs performed with standard samples containing only one of the NT.

The second eluted peak (retention time 2.29 min) was identified as NE, while E eluted around 0.47 min after NE (Fig. 1). The last peak was 5-HT (retention time 5.27 min), when it was located 1.1 min after the elution of D (Fig. 1). Therefore, all four NT could be analyzed in a run of 6 min (Fig. 1).

The number of plates recorded for 5-HT were 61,200; this parameter was 2.8, 5.6 and 8.4 times lower for D, NE and E, respectively (Table 1). The figures recorded for the separation factor and resolution were always higher than 1.4 and 4.4, respectively (Table 1).

The quantification of the NT was performed recording the MS signal in the simple ion mode to gain sensitivity. The most abundant $m/z$ relationships of each NT were selected for this purpose (Fig. 2). Therefore, samples and standards were analyzed by monitoring the $m/z$ relationships of 152, 166, 137 and 160 for NE, E, D and 5-HT, respectively. Fig. 3 displays mass chromatograms of a sample obtained after inducing the secretion of NT with a pulse of $K^+$ according to the procedure described in Section 2.

The lowest detection limit was estimated for 5-HT (0.78 ng/mL), where the detection limits of E, D and NE were a total of 2.4, 8.3 and 12 times higher than the limit of 5-HT (Table 2). The lowest quantification limit was 1.6 ng 5-HT/mL, where this value was 5.3 and 9.4 times lower than the quantification limits of D and NE (Table 2). The quantification limit for E was similar to that of 5-HT (Table 2). All quantification limits were lower than the concentrations of all NT found in basal and potassium-induced secretions by bovine chromaffin cells.

The responses of the MS detector to concentrations of NE and D were linear until 4 and 1 μg/mL, respectively (Fig. 4A and C).

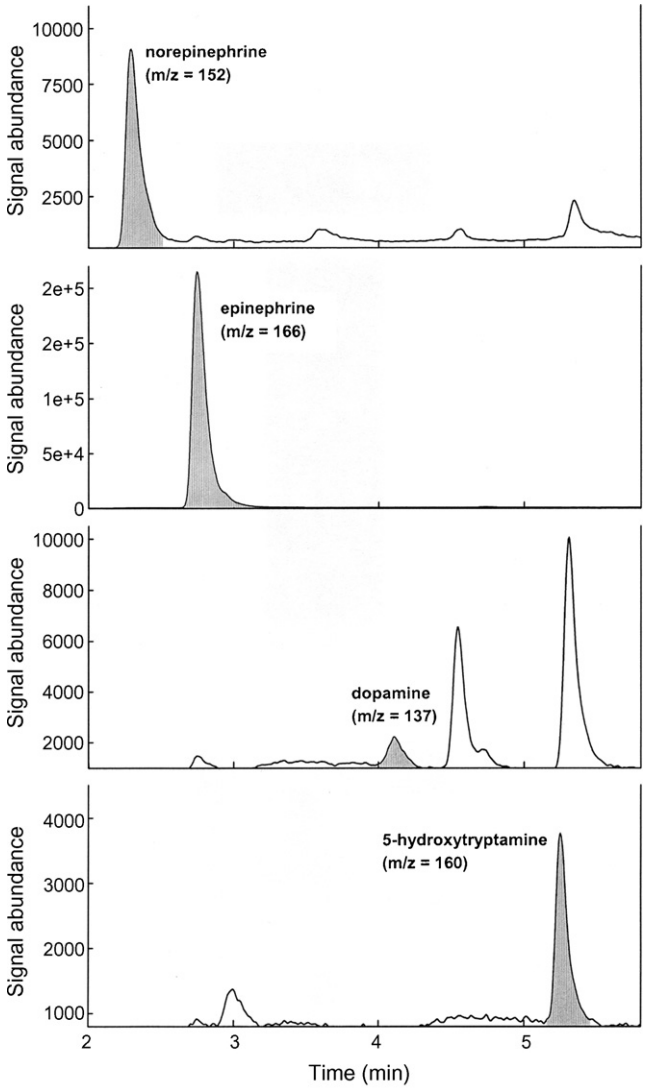


Fig. 3. Chromatographic profiles of a sample of an induced secretion. The secretion was induced with a pulse of potassium in accordance with Section 2.

However, in the cases of E and 5-HT the linearity was kept only from limits of detection to 63 and 250 ng/mL, respectively (inner panels of Fig. 4B and D). These ranges of linearity are suitable for our purposes only in the case of 5-HT since the concentration of E secreted by chromaffin cells in basal situation was higher than 63 ng/mL (data not shown).

Table 2
Analytical data: regression equations, coefficients of regression and detection and quantification limits for the analysed neurotransmitters

| Analyte | Regression equation | $R^2$ | Detection limit (ng/mL) | Quantification Limit (ng/mL) |
|---|---|---|---|---|
| NE | $y = 772000x - 1290$ | 0.999 | 9.6 | 15 |
| E (range between 0 and 4000 ng/mL) | $y = 4410000x^{0.659} - 66000$ | 0.999 | – | – |
| E (range between 0 and 63 ng/mL) | $y = 5900 + 8800000x$ | 0.999 | 1.9 | 2.1 |
| D | $y = 2236000x - 8500$ | 0.999 | 6.5 | 8.4 |
| 5-HT (range between 0 and 4000 ng/mL) | $y = 3870000x^{0.855} - 7900$ | 0.999 | – | – |
| 5-HT (range between 0 and 250 ng/mL) | $y = 5034000x + 2200$ | 0.999 | 0.78 | 1.6 |

Equations of calibration curves display the best mathematical model that fits the experimental data displayed in Fig. 2. Detection and quantification limits were estimated from the signal/noise ratio as described in Section 2.

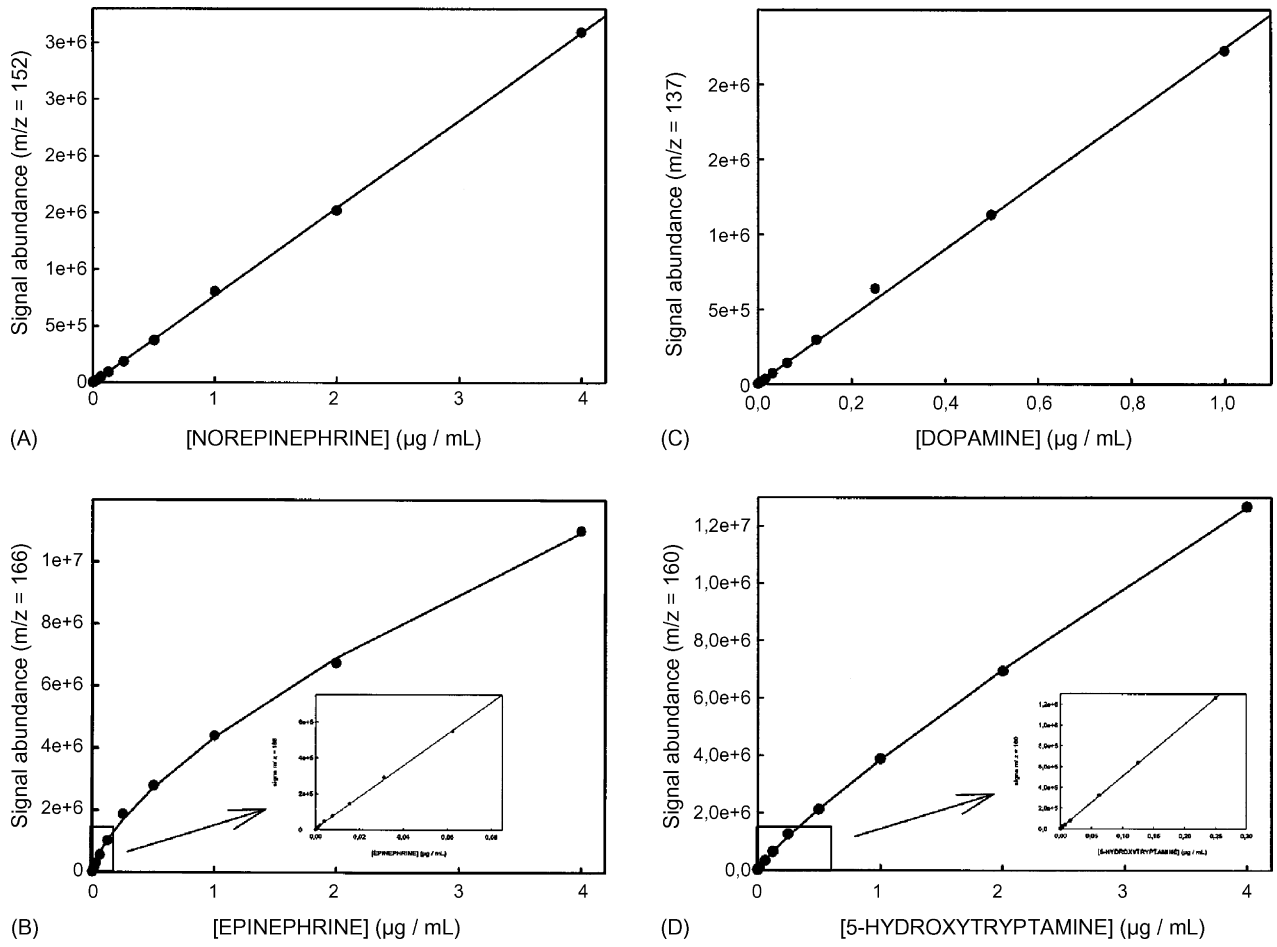


Fig. 4. Standard calibration curves of neurotransmitters. Fourteen different solutions with concentrations ranging between 0.48 and 4000 ng/mL of E, NE and 5-HT and between 0.48 and 1000 ng/mL of D were analysed following the described method. The different solutions were made up by serial dilutions starting from the highest concentration and following a geometric series with a ratio of 2. The areas of *m/z* 152 for norepinephrine (panel A), 166 for epinephrine (panel B), 137 for dopamine (panel C) and 160 for 5-hydroxytryptamine (panel D) were integrated and plotted against the NT concentration. The best mathematical fits to the recorded experimental data are displayed in Table 2. The inner plots of panels B and D display an enlargement of the linear zone.

The response of the MS detector to 5-HT and E in the range between the detection limit and 4 µg/mL was not lineal (Fig. 4B and D). The best mathematical models to fit the experimental data are presented in Table 2. In all cases, the mathematical fits (lineal and non-lineal) displayed a regression higher than 0.999 (Table 2).

Table 3 displays the precision and accuracy data calculated from using the concentrations in the same order of magnitude as the concentrations of each NT, which are typically recorded in basal and induced secretions. Precision ranged between 5% for the highest concentration of D and 23% for the lowest concentration of NE. The lowest accuracy (2%) was found for the highest concentration of E, while the highest accuracy was found for the lowest concentration of 5-HT. All these figures were deemed as satisfactory by considering the well-known variability found among different cultures.

Table 3
Precision and accuracy off the method

| Analyte | Concentration (ng/mL) | Precision (%) | Accuracy (%) |
|---|---|---|---|
| NE | 500 | 23 | 8 |
| | 2000 | 8 | 9 |
| E | 125 | 15 | 12 |
| | 500 | 15 | 2 |
| D | 7.8 | 8 | 9 |
| | 31 | 5 | 3 |
| 5-HT | 8 | 12 | 11 |
| | 31 | 5 | 13 |

The displayed figures were calculated as described in Section 2 for four independent experiments. Dates were calculated from standard solutions.

### 3.2. *Effect of mipafox on the secretion of NT by bovine chromaffin cells*

Table 4 displays the percentage of basal and induced secretion as well as the percentage of intracellular content in relation to the total amount of each NT of each cultured well. Under a control situation, the pulse of $K^+$ induced a secretion 1.8, 2.5, 3.3 and

Table 4
Effect of mipafox on basal and induced secretion and on the intracellular content of neurotransmitters of chromaffin cells

| Neurotransmitter | $n$ | % of total content | | |
|---|---|---|---|---|
| | | Basal | Induced | Intracellular reservoir |
| NE | | | | |
| Control | 24 | 17.1 ± 1.4 | 31.1 ± 2.6 | 51.8 ± 2.6 |
| Mipafox | 12 | 15.0 ± 2.0 | 34.1 ± 4.7 | 51.0 ± 5.0 |
| E | | | | |
| Control | 29 | 21.2 ± 1.1 | 53.0 ± 1.2 | 25.9 ± 1.4 |
| Mipafox | 15 | 21.5 ± 2.0 | 50.0 ± 3.1 | 28.8 ± 2.5 |
| D | | | | |
| Control | 13 | 7.9 ± 1.0 | 26.0 ± 2.9 | 65.9 ± 3.4 |
| Mipafox | 6 | 11.7 ± 2.1 | 34.3 ± 7.9 | 53.8 ± 8.2 |
| 5-HT | | | | |
| Control | 27 | 7.4 ± 1.0 | 19.7 ± 2.1 | 72.7 ± 2.8 |
| Mipafox | 12 | 14.8 ± 4.7* | 27.4 ± 4.1* | 58.3 ± 7.4 |

Cells were treated 60 min at 37 °C with either Krebs buffer (control) or 75 μM mipafox. After removing the mipafox, the secretion was assayed by incubating cell cultures at 37 °C for 5 min with either Krebs buffer (basal) or with Krebs 75 mM $K^+$. The intracellular reservoir of neurotransmitters was assayed after lysating cells with 0.4 M perchloric acid. Neurotransmitters were assayed according to the chromatographic method described. Table displays mean ± standard error for $n$ independent experiments. Statistical significance was analysed using a Student $t$-test.
* Statistically different from control ($p < 0.05$).

2.7 times higher than the basal secretion of NE, E, D and 5-HT, respectively.

No significant differences were observed in both secretions (basal and induced) and the intracellular content of E, NE and D among control cells and those cells treated for 60 min at 37 °C with 75 μM mipafox (Table 4). However, this same treatment of mipafox caused a significant ($p < 0.05$) increment in the basal and induced secretions in relation to the control cells. The increment in the secreted 5-HT also caused a significant ($p < 0.05$) reduction in the intracellular content of 5-HT (Table 4).

## 4. Discussion

The detection limits of NE, D and 5-HT reported by Vaarman et al. [18] for the simultaneous detection of catecholamines, kynurenine and indole derivates of tryptophan by HPLC with electrochemical coulometric detection were 0.65, 0.7 and 1.6 ng/mL, respectively. The values for NE and D were around 10 times lower than the equivalent figures reported in this work (Table 2). On the other hand, the value reported for 5-HT by Vaarman et al. was 2 times higher than the value seen in Table 2.

The detection limits showing Table 2 for NE, E and D were in the same order of magnitude as those limits reported for a method to determine 5-HT and catecholamines in the nervous system of snails using HPLC with an electrochemical detector [19]. On the other hand, the detection limit for 5-HT with this methodology was 15 times higher than those described in our HPLC–MS method.

The quantification limits for a method to analyze catecholamines in urine by ion electrospray tandem MS were 2.5, 10 and 2.5 ng/mL for E, NE and D, respectively [14]. Therefore, the values for E and NE were in the same order of magnitude as the values reported in this work, while D exhibited detection limit 3.4 times lower than those values displayed in Table 2.

The HPLC–MS method presented by Chan and Ho [13] to analyze catecholamines and metanephrines in human urine displayed a detection limit of 5 ng/mL for E, NE and D. These values seem to be in the same order of magnitude as the values estimated by the methodology presented herein.

Therefore, the detection limits displayed by our method are similar to most of the figures reported in the literature. Nevertheless, it is clearly established that the detection and quantification limits estimated for all analyzed NT are compatible with the use of this method to analyze secretions of bovine chromaffin cells. Despite taking detection limits into account, the main advantage of the methodology described in this work is that the injection of the secretions and lysate into a HPLC system is performed with no previous sample treatment. It is also remarkable that the chromatographic method allows four different NT to be analyzed in a run time as short as 6 min.

This methodology should be suitable to determine these NT in other biological fluids, such as human blood. The concentrations of NE, E and D in the blood of a healthy population ranged among 100–350, 20–50 and 25–50 pg/mL, respectively and between 0.1 and 0.2 μg/mL for 5-HT [20]. In this case therefore, the use of this method to determine these NT in human blood would require a previous treatment of concentration.

It has been previously reported that mipafox was able to inhibit 28% and 65% of the catecholamine secretion induced by the pulse of potassium or nicotinic agonist, respectively, through the inhibition of the voltage-dependent calcium channels of bovine chromaffin cells [21]. Other organophosphorus compounds, such as methyl parathion and malathion, were also able to inhibit the activity of these channels [22]. However, we have demonstrated that the incubation of bovine chromaffin cells with 75 μM mipafox for 60 min at 37 °C had no significant effect on the basal and induced secretion and intracellular content of E, NE and D (Table 4). These discrepancies could be explained by the reversibility of the effects of mipafox. Indeed, previously reported inhibitions were detected when calcium currents and catecholamine secretions were recorded in the presence of the organophosphorus compound [21]. In our experiments however, the secretion was induced after washing the cells, therefore in the absence of mipafox.

The meaning behind the increase in the 5-HT secretions after mipafox exposure is currently under investigation in our laboratory. Mipafox is able to induce a delayed poly-neuropathy initiated by inhibition and a further modification of Neuropathy Target Esterase. This protein is a membrane protein located at the cytoplasmic face of the endoplasmic reticulum [23], and therefore its modification might cause alterations in the secretion of NT. By considering that chromaffin cells have proved to be a good cellular model for the study of Neuropathy Target Esterase [2,3], we are also investigating whether the treatment with mipafox might cause some type of alteration in the NT

secretion that could be detectable several hours or days after exposure.

In conclusion, we have described a simple, fast and reliable method for the determination of E, NE, D and 5-HT secreted by cell cultures. The method was based on the direct injection of 25 μL of secretions into a HPLC system with a $C_8$ reversed-phase column coupled with an atmospheric pressure chemical ionization MS detector working in selected ion mode, and was developed with the aim of using the alterations in the secretions of these NT by bovine chromaffin cells as a possible endpoint in the assessment of xenobiotic neurotoxicity.

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