Determination of catecholamines in plasma from rats by direct injection using HPLC

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ABSTRACT

In the present research, essays were made for the determination of catecholamines (nor-epinephrine, epinephrine and dopamine) in plasma. High-performance liquid chromatography with UV detection was employed for quantitative analysis. The method involved direct injection of plasma from rats in a serum albumin dimethyloctyl-silica-gel (HSA-C₁₈) and the utilization of phosphate buffer (pH=6.0):acetonitrile (97.5:2.5 v/v) and 0.01g Heptanosulphonic acid as mobile phase. Identification was based on retention time. Quantification was performed by peak-area determination. The recovery values of catecholamines were 57% to norepinephrine, 71% to epinephrine and 85% to dopamine. The quantification limit is equal to 0.156mg mL⁻¹. The HPLC method proposed here permits good separation of catecholamines on samples of plasma from rats. The method is advantageous due to: high precision and good selectivity.

Key Words: catecholamines, plasma, HPLC

INTRODUCTION

Catecholamines are active amines containing catechol, which act as neurotransmitters and hormones. Such substances are biosynthesized from tyrosine (FIGURE 1).

Catecholamines are controllers of the autonomous and central nervous system (Benedict, 1987). Studies conducted to understand the synthesis and the metabolism of these monoamines have been the main con-
cern of many authors (Scheurink et al., 1989, Kv etnansky et al., 1993, Pappa-Louisi et al., 1997). The determination of catecholamines in plasma or urine by liquid chromatography includes the extraction and purification of catecholamines before injection in the High Performance Liquid Chromatography (HPLC). This way, the use of alumina in acid form has been the extraction method most used to the purification of catecholamines (Kvetnansky et al., 1993, Javidan & Cwik, 1996). The extraction of catecholamines by the on column technique is also reported, linking them to diphenylborates, following by the elution of the catecholamines complexes with a C\textsubscript{18} chromatographic column (Ni et al., 1989). For on line extraction and the determination of catecholamines in urine samples it was used packed chromatographic columns with stationary phase containing borates (Eriksson & Wikström, 1992).

The separation and quantification of small molecules from matrixes formed by macromolecules (drugs, hormones and metabolites) has been a challenge in the field of liquid chromatography. This is due to the need of initially removing the proteins to prevent damage to the chromatographic columns. The conventional preparation of the samples involves procedures of protein precipitation followed by extraction and pre-concentration of analytes. The stationary phases with Internal Surface Reversal Phase (ISRP) were developed by Pinkerton (Pinkerton, 1991). This stationary phase allows the direct injection of the biological fluid sample without any previous treatment.

The aim of this study was to develop a specific analytic methodology using an ISRP chromatographic column, which allows the direct injection of the plasma sample in CLAE, for the determination and quantification of catecholamines.

FIGURE 1 – Biosynthesis of catecholamines.

Enzymes: 1 - Phenylalanine-hydroxilase, 2- Tyrosine-hydroxilase, 3- Amino- decarboxylase aromatic acid, 4- Dopamine-hydroxilase, 5- Phenylethanolamine-N-methyl- transferase.
MATERIAL AND METHODS

Materials

- Reactives
Reactives such as heptanosulphonic acid, epinephrine, nor-epinephrine, dopamine, L-dopa and human albumin were purchased from Sigma-Aldrich Chemical Company (USA). Dibasic sodium phosphate and chloridric acid were obtained from Merck-E. Merck RgaA (Darmstadt, Germany). Acetonitrile (CLAE grade) and EDTA were obtained from Carlo Erba-(Milan, Italy).

Pure water was obtained from a Milli-Q purification system purchased from Millipore, (Millipore, Bedford, MA, USA).

- Instrumentation
The chromatographic analysis was made under isocratic conditions in a high performance chromatography system (Varian Model 2510), equipped with a reciprocate pump; a detector for U-V with variable wave length (Varian Model 2550) adjusted in 205nm and an integrator SP 4400 Chromajet (Varian Associates, Inc; Sunnyvale, CA, USA). It was used a manual system for injection - Rheodyne (7125, Cotati, CA, USA) with sampler of 10mL.

Method

- Source of plasma
It was used adult, male, Wistar albino rats, (UNESP strain), weighing circa 300 g and average 90 days old, furnished by the Central Biocy of the UNESP - Botucatu campus (SP). Animals were anesthetized with sodium pentobarbital. Blood samples were collected in flasks with cateterization of the abdominal aor ta. Immediately after collection, the blood was centrifuged at 2000 R.P.M for 20 min. at 2∞C. The obtained plasma was stored at -20∞C.

- Immobilization of albumin from human plasma - HSA
The chromatographic column was prepared according to the protocol described by Menezes (Menezes et al., 1999), considering a C18 column (150mm x 4.6mm - DI) Phenomenex - Luna 5mm, obtained from Labtron Comércio e Representações Ltda.

- Chromatographic optimization
- Preparation of the standard-solution of catecholamines
The standard-solution were prepared by dissolution of 0.001 g of each catecholamine in 10mL of chloridric acid 0.1mol.L⁻¹. From the mother solution, other dilutions were made attaining concentrations ranging from 0,625mg.mL⁻¹ to 5,0mg.mL⁻¹.
- Chromatographic evaluation after the protein immobilization, (HSA)

The evaluation of the immobilization of proteins over the surface of the stationary phase was done by injecting a sample of the plasma strengthened with catecholamines with a concentration ranging from 1.0mg.mL\(^{-1}\) to 4.0mg.mL\(^{-1}\). The determination of the number of theoretical plates (N), capacity factor (k), peak asymmetry (As) and equivalent height of the theoretical plates (H) were done in a chromatogram obtained after injection of a standard solution containing 2.5mg.mL\(^{-1}\) catecholamines.

- Chromatographic conditions

- Preparation of the analytic curve and strengthening of the plasma sample

The calibration curves were obtained by injection of 10mL of the standard solution containing 0.625, 1.25, 2.5 and 5.0mg.mL\(^{-1}\) of catecholamines.

The plasma sample was diluted in deionized water (1:100) which received amounts of catecholamines to obtain the concentrations of 1.0, 2.0 and 4.0mg.mL\(^{-1}\). These samples were directly injected in the liquid chromatographic system equipped with a chromatographic column ISRP-C\(_{18}\).

- Determination of the limit of quantification

After the selection of the adequate mobile phase it was determined the limit of detection by sampling different and decreasing concentration of catecholamines in order to obtain peaks with height twice as much as to the noise signal.

- Separation of catecholamines in the plasma samples

The separation of catecholamines was made at a constant temperature of 25\(^{\circ}\)C with mobile phase flux adjusted to 1.0mL.min\(^{-1}\). The mobile phase was made with a solution of sodium diacid phosphate 0.05mol.L\(^{-1}\) (pH=6.0) and acetonitrile (97.5:2.5 v/v), containing 0.01g of heptanossulphonic acid.

The experiments for separation of the catecholamines in the plasma samples were made by injection of 5 replicas for each concentration of the plasma sample previously strengthened, containing catecholamines concentrations ranging from 1.0mg.mL\(^{-1}\) to 4.0mg.mL\(^{-1}\). The results obtained were evaluated according the percentage of recuperation and relative standard deviation.
RESULTS

TABLES 1, 2 and 3 show the results obtained in the evaluation of the chromatographic column after the immobilization of HSA, the parameters of the analytical curve of catecholamines and the absolute percentage of recuperation of catecholamines in the rat plasma, respectively.

FIGURE 2 shows a chromatogram obtained after injection of 10mL of a 2,5mg.mL⁻¹ solution of catecholamines (nor-epinephrine, epinephrine and dopamine) after the chromatographic optimization. FIGURE 3 shows a chromatogram obtained after the injection of 10mL of plasma 1:100 strengthened with a standard solution of catecholamines (4,0mg.mL⁻¹) after the chromatographic optimization.

**TABLE 1:** Number of theoretical plates (N), capacity factor (k), peak asymmetry (As) and equivalent height of the theoretical plates (H) of the different catecholamines using a chromatographic column ISRP C₁₈ (150mm x 4.6mm DI).

<table>
<thead>
<tr>
<th>Analite</th>
<th>TR(min)</th>
<th>N</th>
<th>H(mm)</th>
<th>K</th>
<th>As ₀,₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norepinephrine</td>
<td>4.26</td>
<td>2513</td>
<td>5.968</td>
<td>4.325</td>
<td>1.0</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>6.23</td>
<td>1345</td>
<td>2.790</td>
<td>6.787</td>
<td>1.2</td>
</tr>
<tr>
<td>Dopamine</td>
<td>12.72</td>
<td>2490</td>
<td>1.506</td>
<td>14.900</td>
<td>1.0</td>
</tr>
</tbody>
</table>

**TABLE 2:** Equation for the analytical curve of catecholamines

<table>
<thead>
<tr>
<th>Analite</th>
<th>Equation of linear regression</th>
<th>Coefficient of determination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norepinephrine</td>
<td>Y=528.26+3701.00 X</td>
<td>0.996</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>Y=1108.34+4196.75 X</td>
<td>0.992</td>
</tr>
<tr>
<td>Dopamine</td>
<td>Y=1178.08+6262.46 X</td>
<td>0.998</td>
</tr>
</tbody>
</table>

x in mg.mL⁻¹ and y in area units

**TABLE 3:** Absolute recuperation of catecholamines in rat plasma.

<table>
<thead>
<tr>
<th>Analite</th>
<th>Concentration (µg.mL⁻¹)</th>
<th>Absolute recuperation (%)</th>
<th>Relative standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nor-epinephrine</td>
<td>4.0</td>
<td>82.35</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>68.00</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>57.00</td>
<td>5.2</td>
</tr>
<tr>
<td>epinephrine</td>
<td>4.0</td>
<td>89.30</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>81.00</td>
<td>17.0</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>71.30</td>
<td>18.0</td>
</tr>
<tr>
<td>Dopamine</td>
<td>4.0</td>
<td>93.30</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>85.00</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>87.30</td>
<td>8.0</td>
</tr>
</tbody>
</table>
**FIGURE 2:** Chromatogram obtained after the injection of 10mL of a 2.5mg.mL^{-1} solution of catecholamines (nor-epinephrine, epinephrine and dopamine), using the mobile phase constituted of a mixture of a solution of sodium diacid phosphate 0.05mol.L^{-1} with pH adjusted to 6.0, acetonitrile (97.5:2.5 v/v) and 0.01g of heptan sulphonic acid, flux of 1.0mL.min^{-1} and detection at 205nm.

Peak: 1 - nor-epinephrine, 2 - L-dopa and 3 - dopamine.

**FIGURE 3:** Chromatogram obtained after the injection of 10ml of a sample of diluted plasma at 1:100 plus a solution of catecholamines (nor-epinephrine, epinephrine and dopamine) at 4.0mg. mL, using the mobile phase of a mixture of a solution of sodium diacid phosphate 0.05mol.L with pH adjusted to 6.0, acetonitrile (97.5:2.5 v/v) and 0.01 of heptasulphonic acid, flux of 1mL.min^{-1} and detection 205nm.

Peak: 1 - Plasma, 2 - nor-epinephrine, 3 - L-dopa, 4 - dopamine.
DISCUSSION

The new chromatographic column allows the separation and determination of catecholamines by direct injection of the strengthened samples in CLAE.

The chromatographic column performance was evaluated in terms of retention of time and the parameters presented in Table 1 (number of theoretical plates (N), capacity factor (k), peak asymmetry (As) and equivalent height of the theoretical plates (H) of the various catecholamines), calculated after Figure 2. Such results are considered satisfactory since the immobilization of HSA over the surface of the external surface of the spherical particles of the stationary phase C18 has helped to obtain well-developed peaks and separation of catecholamine in the plasma in the following periods of the reaction: 4.35, 6.38 and 13.18 minutes for nor-epinephrine, epinephrine and dopamine, respectively.

According to the literature, coefficients of peak asymmetry ranging from 1.0 to 1.5 are considered excellent (Ciola, 1989). Therefore, the peak asymmetry coefficients for catecholamines calculated (Table 1) reveals an adequate performance of the chromatographic column ISPR, in which the immobilization of HSA was done. According to these values, it is possible to state that this chromatographic column has an excellent chromatographic performance.

The coefficients of determination (Table 2) show that the dilution of catecholamine is replicable using the described chromatographic optimization.

During the chromatographic optimization, various compositions of the mobile phase were tested. However, the most adequate mobile phase was constituted by the solution of sodium phosphate diacid 0.05mol.L\(^{-1}\) (pH=6.0) and acetonitrile (97.5:2.5 v/v), containing 0.01g of heptanossulphonic acid.

The presence of heptanossulphonic acid in the mobile phase is mandatory since this compound forms an ionic pair with catecholamines making possible, therefore, the retention of these amines in the ISRP stationary phase. In this way, this compound has contributed greatly to the separation of the catecholamines. Figure 3 shows a chromatogram obtained after the injection of 10mL of plasma 1:100 strengthened with a pattern solution of catecholamines in which the optimized mobile phase allows a stable baseline during the separation of the catecholamines. It is important to stress that the plasma sample as diluted 1:100 in order to assure that metabolites and small concentration of proteins were quickly eluted out of the chromatographic column. However, it was observed that the catecholamines were extracted in the stationary phase without interfering peaks.

The chromatographic studies to evaluate the efficiency of the extraction were tested by the strengthening of the rat plasma samples with cat-
echolamines, as concentrations of 1.0, 2.0 and 4.0mg.mL⁻¹ were injected in 5 replicates. The results obtained are satisfactory (TABLE 3) since the recuperation of catecholamines in strengthened rat plasma was greater than 57.71 and 85% for norepinephrine, epinephrine and dopamine, respectively. It is important to note that the method is simple, rapid, cheap and reproducible.

The limit of quantification determined was 0.156mg.mL⁻¹ for all catecholamines. One should remember that the U-V detector was adjusted to a wavelength of 205nm, being this the most sensitive to the detection of catecholamines. The band has varied between 0.08 and 0.02 and the attenuation from 2 to 1. In this regard, the linearity of the detector was determined evaluating relations among concentrations from 0.625 to 5.0mg.ml⁻¹.

Recent literature has reported the use of a pre-column C₈ (30mm×4.6mm, DI) for the pre-concentration of the sample and to reduce the influence of the matrix (Raggi et al., 1999). Therefore, the proposed analytical method does not require the preparation of the plasma sample before its analysis, making this a simple and rapid method to the determination of catecholamines.

CONCLUSION

The new method proposed to the determination of catecholamines using an isocratic liquid chromatographic system equipped with visible U-V detector and the application of a chromatographic column ISRP-C₁₈ made possible the development of a simple, rapid and precise methodology to proceed to the separation and determination of catecholamines present in plasma samples.

ACKNOWLEDGEMENTS

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BIBLIOGRAPHIC REFERENCES


