The Determination of Paracetamol by HPLC Validation of the Method and Application on Serum Samples

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Pain is defined as a disagreeable sensory and emotional experience related to a tissue or potential lesion. Paracetamol (Acetaminophen) is the most used non-morphine analgesic. For the determination of paracetamol we developed and validated the high performance liquid chromatography (HPLC) analysis using a Dionex Ultimate 3000 liquid chromatograph equipped with a multidimensional detector. After determining the optimum conditions of analysis (80/20 water / acetonitrile mobile phase, flow rate 1.0 mL / min, detection wavelength 245 nm) we validated the method following the following parameters: linearity of response function, linearity of results, limit (LD = 0.66 µg / mL) and quantification limit (LQ = 2.00 µg / mL), and precision. The method of determining paracetamol by HPLC was applied to 30 samples of serum collected from patients who had pain and were treated with paracetamol.

Keywords: paracetamol, HPLC, serum

Paracetamol (Acetaminophen) is the most used non-morphine analgesic. Acetaminophen in the critical patient is limited to the treatment of moderate-intensity or antipyretic pain. It was synthesized by acetylation of para-aminophen with acetic anhydride. It is a white to off-white, crystalline powder, bitter, slightly soluble in alcohol, soluble in acetone and alkali, poorly soluble in water [1, 2].

Numerous methods for determining paracetamol from both different pharmaceutical forms and biological fluids [2, 3] are described in the medical literature.

In our study, an HPLC method for the determination of paracetamol applied to serum samples from patients diagnosed with pain and treated with injectable paracetamol is developed and validated.

The proposed method was elaborated and validated in order to establish the chromatographic parameters recommended in the medical literature [2, 10].

Experimental part
Development and validation of the HPLC method for the determination of paracetamol

Material and method
- Dionex Ultimate 3000 Liquid Chromatograph equipped with multidimensional detector
- Eclipse XDB C18 column (150 mm x 4.6 mm; 5 µm)
- Kern 770 analytical scale
- Ultrasonic bath MTH 2510
- Chromatographic purity acetonitrile (Merck)
- Chromatographic Purity Methanol (Merck)
- Paracetamol (standard of work) - (Zentiva Company)
- Standard solutions are obtained by diluting with methanol
- Column temperature 25 ºC.
- Mobile phase: water / acetonitrile mixture in 80/20 ratio.
- Mobile Phase Flow: 1 mL / min.
- Volume of solution injected: 20 µL.
- Detection is performed at a wavelength of 245 nm.

Sample preparation
Precipitation of the serum proteins was performed with methanol. After stirring vigorously for 5 min, the mixture is centrifuged and a volume of 20 µL is taken from the clear supernatant which is analyzed under the conditions of the method.

Working method
After establishing the detection wavelength (maximum peak absorption measured at the very peak obtained in the chromatogram of a standard solution of paracetamol, λ = 245 nm) the paracetamol was identified from the chromatograms of the sample solutions by comparing the retention times (for the chromatogram of the standard solution respectively for the sample) and UV absorption spectra (fig.1).

Fig. 1. Chromatogram of paracetamol - standard solution and sample solution

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The linearity of the response function was studied (the peak area was modified based on the injected concentration). After statistical processing of the experimental data it is observed that on the chosen working field (0.02 - 20 µg / mL) the method has two lines of linearity: 0.02 - 12.75 µg / mL (the equation of the regression line is Peak area = 1.8229 x Concentration (µg / mL) - 0.3343, regression coefficient, r² = 0.9982, standard error of regression curve, SE = 0.3650) and 12.75-20 g / mL (equation of regression line is Peak area = 3.0865 x Concentration (µg / mL) - 16.452, regression coefficient, r² = 0.9921) (fig. 2).

![Fig. 2. Calibration Straight Line for Paracetamol Determination by HPLC](image)

The detection limit (LD = 0.66 µg / mL) and the limit of quantification (LQ = 2.00 µg / mL) were calculated using the estimation of these limits based on the standard deviation and the slope of the regression line.

To estimate accuracy, we determined:
- Repeatability of the injection (system precision) for a number of 10 determinations, the relative standard deviation (RSD) being 0.7524%.
- Repeatability of the analysis/test (precision of the method) for six independent solutions at a concentration of 10 µg / mL, for which the relative standard deviation (RSD) is 3.3257%.
- Intermediate precision for six independent solutions at a concentration of 10 µg / mL, for which the relative standard deviation (RSD) is 4.2468%.
- For the estimation of accuracy, a retrieval was determined for three samples at three different concentration levels (80-120%) to obtain an average relative standard deviation (RSD) being 0.7524%;
- For areas smaller than 22.91 (corresponding to a concentration of 12.75 µg / mL) the method has two lines of correlation, and the peak area corresponding to paracetamol is used to calculate the concentration in paracetamol as follows:

- For areas smaller than 22.91 (corresponding to a concentration of 12.75 µg / mL) the following equation will be used:
  - Concentration (µg / mL) = (Area + 0.3343) / 1.8229
- For areas larger than 22.91 (corresponding to a concentration of 12.75 µg / mL) the following equation will be used:
  - Concentration (µg / mL) = (Area + 16.452) / 3.0865

Results and discussions

Correlation of HPLC data with the evolution of pain in the patients studied.

We have dosed the serum paracetamol, as seen in the tables and equations above and we obtained two therapeutically important values:

- Peak area (peak of analgesic action)
- Final concentration (completion of analgesia)

These values were correlated both with the time when paracetamol was administered and with the evolution of pain intensity before and after paracetamol administration, an evolution that we quantified through a simple numerical scale from SNS = 10 maximum unimaginable and SNS = 1 absence of pain [4].

Most of the patients in the study who received paracetamol as the primary analgesic (16.66%), experienced pain only in an anatomical segment: isolated contusions, isolated arthralgia and intercostal neuralgia.

Some were clinically and paraclinically diagnosed with fractures, multiple contusions, abdominal colics (13.33%) and only 10% of those questioned with pain had renal colic or acute limb ischemia, knowing that in such cases the pain is atrocious upon arrival in the emergency service. (fig. 3) [5, 6, 9].

![Fig. 3. Batch structure on recorded diagnostics](image)

Prior to administering any type of painkiller, pain intensity was measured by a simple numerical scale (SNS) from 1 (absence of pain) to 10 (maximum unimaginable pain), each patient quantifying it subjectively.

If before administering paracetamol we notice that most of the patients had unbearable pain (SNS = 9 to 86.7%), as shown in figure 4, we cannot notice the same fact after paracetamol administration. Prior to and after paracetamol administration, the intensity of patients’ pain was evaluated using the single verbal scale (SVS), (SVS = 4 maximum intensity and SVS = 0 absence of pain).

The serum samples were obtained by means of centrifugation. After deproteinization with methanol, a volume of 20µL of the sample was taken from the supernatant and analyzed under the conditions of the validated HPLC method.

Calculation of paracetamol concentration in serum samples

The presence of the peak corresponding to paracetamol was identified by comparing the retention time (2.289 - 2.530 min) and spectral comparison. We integrate the chromatogram, and the peak area corresponding to paracetamol is used to calculate the concentration in paracetamol as follows:

- For areas smaller than 22.91 (corresponding to a concentration of 12.75 µg / mL) the following equation will be used:
  - Concentration (µg / mL) = (Area + 0.3343) / 1.8229
- For areas larger than 22.91 (corresponding to a concentration of 12.75 µg / mL) the following equation will be used:
  - Concentration (µg / mL) = (Area + 16.452) / 3.0865
interfere with an anxious component, and in that case if we only treat pain and/or anxiety?

The time interval for the quantification of pain progression following paracetamol administration was variable (between 30 min and over 60 min) since the patients require in addition to investigations and treatment in emergency services, investigations in other departments (surgical consultation, computer tomography).

The French specialists also faced this inconvenience and have studied pain further, saying that the difficulty of re-evaluating patients in emergency departments is related to the permanent arrival of new patients requiring other therapies, and their monitoring is difficult to insure [5, 9].

After the administration of the second analgesic, we observed a distribution of patients on the lower numerical range (SN5 = 3 to 45.5% of those studied), highlighting the fact already confirmed in medical articles that paracetamol remains a co-analgesic in unbearable pain [6, 8].

Following acetaminophen administration, blood samples were collected on the next subject inquiry which took place between 30 and 60 min and even more, by attempting to quantitatively correlate the presence of paracetamol in the blood and the pain score at that time.

Pharmacokinetic and pharmacological data for acetaminophen show that the plasma peak is reached in 25 minutes after the end of IV of 1 g of paracetamol.

In order to correlate the peak area and the final concentration, namely the time to the first evaluation and the three pain intensity values, we used a linear regression model and we determined the Pearson correlation coefficients.

<table>
<thead>
<tr>
<th>Table 1</th>
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<tr>
<td><strong>STATISTICAL CORRELATION BETWEEN PEAK AREA AND PAIN VALUE</strong></td>
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<tr>
<th>Pearson correlation coefficient</th>
<th>Equation of the line of regression $Y = A \cdot X + B$</th>
<th>ANOVA test for filtering the model</th>
<th>CONCLUSION</th>
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</thead>
<tbody>
<tr>
<td>R</td>
<td>R²</td>
<td>Estimatie error $Y_{\text{est}}$</td>
<td>F</td>
</tr>
<tr>
<td>Peak area vs. Time until assessment</td>
<td>0.139</td>
<td>0.019</td>
<td>51.347</td>
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<tr>
<td>Peak area vs. Value at first assessment</td>
<td>0.060</td>
<td>0.004</td>
<td>0.351</td>
</tr>
<tr>
<td>Peak area vs. Value at second assessment</td>
<td><strong>0.409</strong></td>
<td><strong>0.168</strong></td>
<td>0.990</td>
</tr>
<tr>
<td>Peak area vs. Value at third assessment</td>
<td>0.186</td>
<td>0.035</td>
<td>0.879</td>
</tr>
<tr>
<td>Final concentration vs. Time until first assessment</td>
<td>0.131</td>
<td>0.017</td>
<td>51.406</td>
</tr>
<tr>
<td>Final concentration vs. Value at first assessment</td>
<td>0.045</td>
<td>0.002</td>
<td>0.352</td>
</tr>
<tr>
<td>Final concentration vs. Value at second assessment</td>
<td><strong>0.419</strong></td>
<td><strong>0.176</strong></td>
<td>0.985</td>
</tr>
<tr>
<td>Final concentration vs. Value at third assessment</td>
<td>0.145</td>
<td>0.021</td>
<td>0.885</td>
</tr>
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</table>
Although after the administration of acetaminophen we were unable to question the patients exactly when the maximum plasma concentration (15 min after the 1 g perfusion) was reached, the data in Table 1 confirms a statistically significant correlation between the peak area and the pain score after perfalgan IV perfusion (Fig. 7). Additionally, this moderate correlation, directly proportional to the area of the peak, and the reduction in pain intensity, asserts that the pain self-assessment by the patient is not only subjective.

In the United States Journal of Surgery (2015), it is recommended that acetaminophen be administered preoperatively for its maximal postoperative efficacy, one hour after administration, analgesia occurs and may last for up to 6 h [11].

We find a significant correlation between the final concentration and the second value of pain, which confirms the effectiveness of the treatment and confirms the data from the medical literature (Fig. 8). Even if the antalgic and antipyretic activity is maximal after one hour from the plasma peak, in our study we reviewed and reinterpreted the subjects at more than one hour, which shows that analgesia was maintained in cases of medium and small intensity pain.

Even though paracetamol is also an antalgic good in addition to the antipyretic effect it does not inhibit pain but it alleviates it as shown in Figure 9.

In some patients, it was necessary to introduce a second drug to annihilate the pain.

To compare the scores obtained in the first, second and third pain assessment, we used the marginal homogeneity test designed specifically for category variables with more than two categories, such as pain intensity assessment scores, comparison that clearly demonstrates the effectiveness of paracetamol as an analgesic. If at the first evaluation the maximum score (SNS = 9) had a majority percentage (86.7%) at the last interrogation of the patients, the percentage was evenly distributed and the prevailing score was minimal (SNS = 3 in 45.5% of the subjects).

This time there is only a correlation between paracetamol dosing and the final value, explained by the presence of another analgesic in the patient’s serum and the absence of acetaminophen, in most cases the symptomatology not being there anymore.

Conclusions

Both the theoretical and practical studies on 30 patients who presented themselves in the pain emergency service confirm that paracetamol remains a unique painkiller in moderate to low pain and co-analgesic pain in severe pain. Significant correlations between the paracetamol plasma concentration and pain assessment scores confirm the efficacy of acetaminophen as an analgesic.

Due to the fact that there were no adverse reactions in the subjects studied, we can say that paracetamol can be used as single dose antalgic even in patients with hepatic dysfunction.

Considering some patients, need to add an opioid co-analgesic or an anti-inflammatory, the question remains whether paracetamol is effective only in patients with minimal or moderate pain, or the dose given at the beginning should be bigger as the French specialists have confirmed.
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