UNIVERSITY OF OVIEDO

FACULTY OF CHEMISTRY

QUALITY CONTROL OF ANTI-FLU MEDICINES CONTAINING PARACETAMOL AND OTHER ACTIVE INGREDIENTS THROUGH HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

BACHELOR THESIS IN CHEMISTRY

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1. Introduction

1.1. General aspects of Paracetamol

Paracetamol (PCT), also known as acetaminophen, N-acetyl-p-aminophenol or 4-acetamidophenol, is a common medication firstly synthesized by the French chemist Charles Gerhardt in 1852, and later on by Harmon Northrop Morse by reduction of p-nitrophenol with tin in glacial acetic acid in 1877. It was not introduced to medicine until 1893, when the pharmacologist Joseph Von Mering reported the first tests of PCT in humans. However, the results of this study concluded that PCT caused methemoglobinemia. PCT remained silent for the following 50 years, when in 1949 several researchers suggested that the disease was caused by other metabolite and PCT could rejoin the medical market[1].

It is used as an analgesic for home medication and is considered an effective treatment for the relief of pain and fever, and one of the most used medicines in the world. It is present in many prescription analgesics and flu medications.

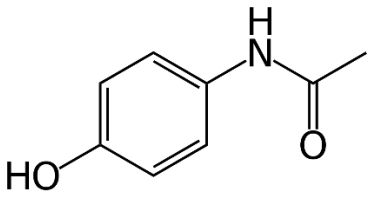

Figure 1. Structure of paracetamol.

In contrast with other common analgesics like ibuprofen or aspirin, PCT is not an anti-inflammatory, and for that reason is not included in the group of non-steroidal anti-inflammatory drugs (NSAIDs). PCT does not affect blood coagulation or irritate the stomach in regular doses, and unlike opioid analgesic, does not cause mood changes or euphoria, and neither create addiction or dependance.

In the pharmaceutical industry, PCT is obtained from the acetylation of para-aminophenol. This reactant could be obtained by nitration of chlorobenzene, followed by hydrolysis under alkaline conditions (and subsequent acidification) and hydrogenation of the corresponding para-nitrophenol (Route 1). In an alternative approach para-nitrophenol is prepared by direct nitration of phenol (Route 2). In both cases, the nitration leads to a significant proportion of the unwanted ortho-isomer that could not be avoided. Other possibility involves the hydrogenation of nitrobenzene to phenylhydroxylamine that is transformed under acidic conditions to the desired para-aminophenol via a Bamberger rearrangement (Route 3)[2].

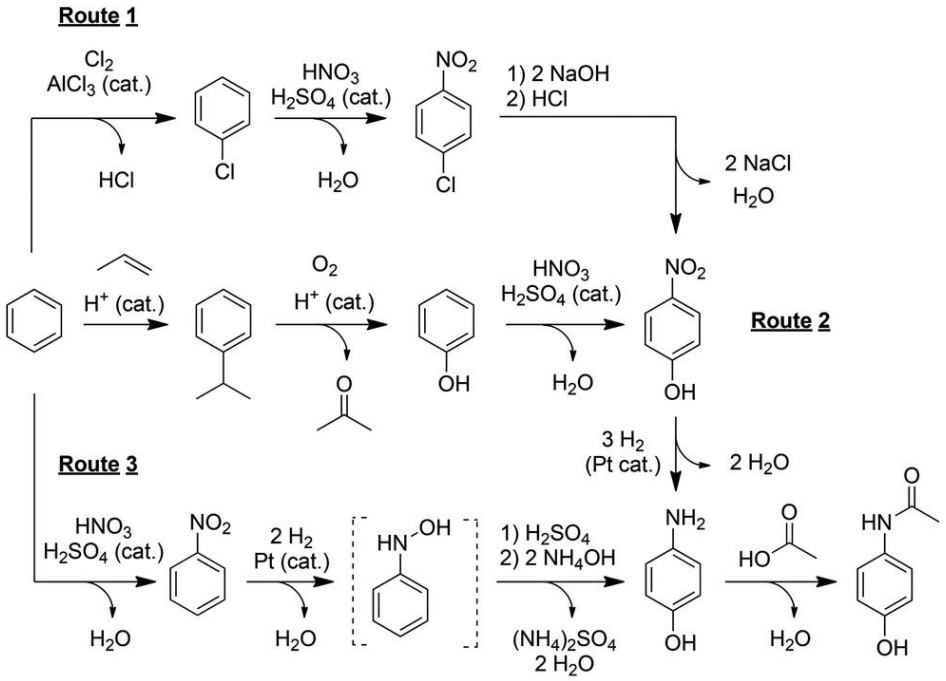

Figure 2. Commercial routes for paracetamol production.

Despite of a relative innocuous character, high PCT levels in the blood flow can lead to hepatic toxicity, if concentration in plasma exceeds 120 µg/mL after 4 hours of the ingestion, and more serious acute damage, if these levels are over 200 µg/mL. This occurs because of the accumulation of toxic metabolites that can cause the death of hepatocytes. If the overdose is prolonged in a period of time, this can lead to an hepatic failure.

PCT is mainly metabolized in the liver by oxidation, reduction, and hydrolysis reactions. The resultant metabolites contain polar groups leading either to activation or inactivation of the drug. Afterwards, conjugation reactions occur between the polar functional groups of metabolites produced before with conjugation groups. Glucuronidation is believed to account for 52–57% of the metabolism of paracetamol, with a lesser contribution of sulfation (30–44%) and oxidation (5%). Glucuronidation and sulfation yield final products that are inactive, nontoxic, and eventually excreted by the kidneys. In the oxidation pathway, however, the hepatic cytochrome P450 enzyme system metabolizes paracetamol, forming catechol and NAPQI (N-acetyl-p-benzo-quinone imine). NAPQI is toxic and is primarily responsible for the previously described hepatotoxicity effect of paracetamol. A fourth pathway accounting for around 6% of paracetamol metabolism was described few years ago. By deacetylation in the liver, PCT yields para-aminophenol (p-aminophenol, 4-aminophenol), which is conjugated with arachidonic acid in the brain using the fatty acid amide hydrolase enzyme (FAAH) to form N-arachidonoylphenolamine (AM404)[3]. The toxicity induced by NAPQI involves two mechanisms: A

covalent binding to hepatic proteins, causing damage to cell membranes and mitochondrial dysfunction, and depletion of glutathione, making hepatocytes more susceptible to a reactive oxygen species induced apoptosis.

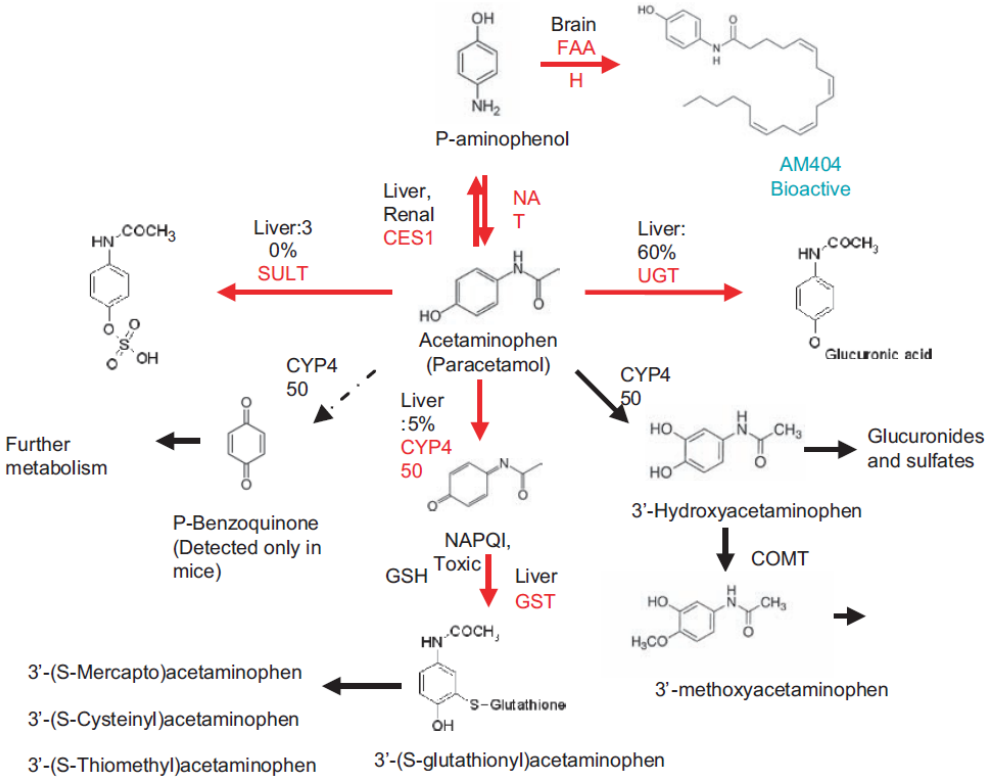

Figure 3. Paracetamol metabolic pathways[3]. Red arrows indicate the main pathways of paracetamol pharmacodynamics and toxicity. Benzoquinone metabolites and AM404 have been detected only in mice.

AM404: N-arachidonoylphenolamine; CES1: Carboxylesterase 1; COMT: Catechol-O-methyltransferase; CYP450: Cytochrome P450 ; FAAH: Fatty acid amide hydrolase enzyme; GSH: Glutathione; GST: Glutathione S- transferase ; NAPQI: N-acetyl-p-benzo-quinone imine; NAT: N-acetyltransferase; UGT: UDP Glucuronosyltransferase; SULT: Sulfotransferase.

Several methods have been used in the determination of PCT in commercial formulations, such as titrimetry, UV/Vis spectroscopy, near infrared spectroscopy and other methods like several flow injection methods have been also proposed in recent years, coupled to an UV/Vis spectrophotometer or a fluorimeter detector. Nevertheless, some of the previously mentioned methods are not convenient for the analysis of PCT in commercial formulations, because they require the hydrolysis of PCT to 4-aminophenol and later the production of a coloured complex[4]. This can lead to a higher reagent consumption, affecting the sensitivity of the method, and also increasing both the analysis time and the possibility of contaminating the sample.

When analysing mixtures with more than one analyte of interest, such as many drugs containing several active ingredients the main problem that has to be solved is the selectivity of the analysis without a proper chemical separation.

In the case of PCT analysis related to biological fluids like blood or urine, fewer methods are found in the available literature. They are usually performed with chromatographic or electrophoretic techniques, but also spectrophotometric and fluorimetric methods have been occasionally used.

1.2. Spectrophotometric methods

Diving more deeply in the methods available, the visible range optical methods usually involve a tritimetric reaction[5] to transform PCT into a coloured molecule. For example, reaction between PCT and p-dimethylaminobenzaldehyde in 2M HCl after applying heat to form a yellow molecule[6] or the hydrolysis with sulfuric acid followed by the diazotisation of the p-aminophenol obtained and lastly a coupling step with β-naphthol in alkaline medium. The main drawback of these methods is that the hydrolysis step is almost always needed and increases the time spent in the analysis in a large extent.

As PCT can be intrinsically detected by UV absorption spectrophotometry, it could be determined by the direct measurement. However, in pharmaceutical formulations the compound of interest is usually mixed with other active substances and excipients that can cause spectral interferences. For this reason, the detector must be coupled with a previous separation technique, such as high-performance liquid chromatography (HPLC).

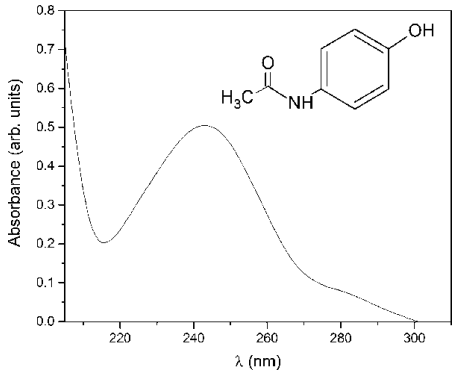

Figure 4. Absorption spectra of paracetamol in de-ionized water[7].

In Table 1 the information related to several UV-Vis spectrophotometric methods for the determination of PTC are presented.

Table 1. Reagents and related methods for the determination of paracetamol with UV-Vis spectrophotometry.

Reagent	Method
3-Methyl-2-benzothiazolinone hydrazone in the presence of ceric ammonium sulphate[8]	In alkaline medium and extraction into CHCl3; λ = 535 nm
Ce(IV)[4]	For 90 min, in concentrated sulphuric acid in a water bath at 80°C; λ = 410 nm
o-Cresol[9]	-
Sodium 1,2-naphthoquinone-4-sulphonate and cetyltrimethyl ammonium bromide[10]	In alkaline medium; determination of hydrolysis products of paracetamol and phenacetin; λ = 570 and 500 nm, respectively
Sodium sulphide and Fe(III) or Ce(IV)[11]	At ambient temperature; determination of p-aminophenol and acetaminophen by formation of a methylene blue like dye; λ = 550 nm
Pyrochatechol violet[12]	Under basic conditions to form a coloured ion-pair complex; λ = 652 nm
Bromate–bromide mixture[13]	Kinetic analysis; generation of bromine; determination of paracetamol and acetylsalicylic acid in mixtures; applying partial least-squares regression to the kinetic photometric data
S^{2-} in the presence of Fe(III)[14]	Microwave assisted alkaline hydrolysis; λ = 540 nm; in only 1.5 min under radiation power 640W
Ammonium molybdate[15]	In strongly acidic medium to produce molybdenum blue; λ = 670 nm
Ammonium molybdate[16]	λ = 695 nm
3-Cyano-N-methoxypyridinium perchlorate+methoxyethanol+chloramine T[17]	λ = 448 and 476 nm
pH-induced spectral changes[18]	λ = 262.5 nm
4-Nitrosoantipyrine[19]	Heating in strongly alkaline medium give a red colour; λ = 515 nm
Tris(2,2_-bipyridine)–iron(III) complex in acid medium[20]	Stopped-flow method for the determination of paracetamol and oxyphenbutazone
Absorbance difference method[21]	Simultaneous and separate estimation of paracetamol and diclofenac sodium; λ = 230 and 254 nm and λ = 260 and 292 nm, respectively

Some reports also show the application of flow-injection analysis systems (FIA) with colorimetric detection, in a similar way to those methods treated before, but in a continuous way. This methodology has the same limitation that was observed in visible spectrophotometry: A hydrolysis step that needs high temperatures is required to obtain a detectable molecule. For this reason, the flow reactor should be inside an oven or similar heat source, and the flow that exits this reactor should be cooled before the detection, increasing the complexity of the system.

The main drawback of the analysis with spectrophotometric techniques is the determination of several compounds present in the mixture without a preliminary separation step. For this reason, several methods such as first or second derivatives or HPLC are applied to mixtures of compounds with overlapping spectra. This helps to increase the sensitivity of the analysis and broaden its utility.

Some active formulations that can be present in tablets or syrups and could interfere in PCT determination are acetylsalicylic acid[22–25], analgine[26], ascorbic acid[24], caffeine[25,27–29], ibuprofen[30–32], metamizol[29], methocarbamol[33,34], phenprobamate[35], propacetamol[36] or propyphenazone[27]. Same considerations must be taken into account with excipients such as colorants[37].

Another interesting spectroscopic technique that can be applied to PCT determination is near-infrared (NIR) spectroscopy[38,39]. This method allows to perform the analysis in a non-destructive manner, without solvents or other reactants, which simplifies the determination. The main problems related to the direct application of NIR to the analysis of tablets is that it is harder to make reproducible sample measurements and the fact that those measurements are made by reflexion and for this reason the information obtained corresponds to the surface of the tablet. With the most recently developed NIR instrumental the measurement method changed to a transmission measurement, where the beam crosses the whole sample. This helps to increase the reproducibility of the measurements and the calibration of the instrument is simpler, because the absorbance signal can be related with concentration following the Lambert-Beer law. However, the transmittance method also brings a new problem, which is the small amount of light that reaches the detector, due to the thickness of the sample and this can affect the sensitivity. One of the major disadvantages of NIR is the strong band overlap of signals, and for this reason, it requires the use of multivariate chemometric techniques.

Spectrofluorimetric methods have been proposed for the analysis of paracetamol showing very low detection limits. PCT itself is not a fluorescent species, so the analysis is determined indirectly by the addition of Ce(IV)[40], which oxidizes PCT and the signal measured is due to the produced Ce(III) in diluted sulphuric acid. The direct determination of PCT requires a previous derivatisation step[41,42], but the selectivity achieved is not very high. The required oxidation step increases the time required for the analysis and also reagent consumption plus the possibility of contamination of the sample.

The last spectrophotometric technique that could be applied to PCT determination is chemiluminescence (CL), due to its high sensitivity and low detection limits. The reaction can proceed either to the formation of a CL active species or the inhibition of the CL for a reactant consumed. Some of the methods described in literature are the oxidation with Ce(IV) in acidic solution[43], the oxidation by iron (III) in the presence of a chelating agent (2,4,6-tris(2-pyridyl)-s-triazine)[44] or the oxidation with hexacyanoferrate(III) and subsequent inhibition of the reaction between luminol and hydrogen peroxide[45]. However, the limit of detection (LOD) of these methodologies is not good enough for the standards of CL analysis.

1.3. Electroanalytical methods

In general, the time consumed by spectrophotometric analysis is something to take into account, as well as the instruments needed and sample preparation processes. Electroanalytical methods create a solution for many of the problems aroused by previously mentioned methods.

A common electroanalytical technique is voltammetry, that can be used in combination with disposable sensors to perform the analysis in a simple and low-cost way, while also being able to be miniaturized. The drawback of voltammetry is that any species that can exchange electrons with the electrode will create an interference, but this can be overcomed by the inclusion of a selective modifying agent in the sensor.

As previously mentioned, PCT can be oxidised directly in a carbon paste electrode, but this process is nonselective and must be carried at a potential between 0.6 and 0.8V, at which other substances are electroactive as well. A way to solve this is treating the graphite electrode with a modifyer, such as N-acetyl-p-benzoquinoneimine[46] merged in the electrode surface through several potential cycles, and after that through an adsorptive transfer stripping differential pulse voltammetry (ATSDPV) the PCT concentration can be determined. Another option is the use of a molecularly imprinted polypyrrole modified graphite electrode[47] treated with cyclic voltammetry as well in a buffer solution that contains PCT later on extracted to create a surface that retains its shape, favoring adsorption.The analysis is performed by differential pulse voltammetry, and this electrode avoids the interferences caused by ascorbic acid, dopamine, glucose, phenacetin or phenol that could be present in the sample.

Amperometry, can be used to determine paracetamol in liquid chromatography by measuring the product of the enzymatic hydrolysis of PCT instead of the PCT itself[48]. The oxidation potential of the product, p-aminophenol, is lower than for PCT so fewer interfering compounds get oxidised as well.

These electroanalytical techniques can be used in combination with biosensors, that increase selectivity and sensitivity using an enzyme or antibody to obtain a new signal proportional with the analyte concentration and without interferences.

Other electroanalytical methods that can be used are ion-selective electrodes, such as fluoride-selective electrode[49], where PCT reacts with 1-fluoro-2,4-dinitrobenzene (FDNB), a potential scanning voltammetric detection[50] or the use of some redox couples as indicating systems for the determination of PCT in a flow injection system through biamperometry[51].

1.4. Chromatographic methods

The simultaneous determination of the active ingredients in multicomponent pharmaceutical products often requires the use of a separation technique, such as gas chromatography (GC) or high-performance liquid chromatography (HPLC).

HPLC is the most popular chromatographic method for separating mixtures of active ingredients in drugs and other excipients as well. The selected detection system must be versatile and be able to detect all of the desirable compounds while keeping selectivity to correctly identify the different species.

A common method to perforrm HPLC analysis of analgesics is to employ reversed-phase columns, such as C18 or cyano columns.

Some procedures developed allow us to determine simultaneously paracetamol, pseudoephedrine and chlorpheniramine in chewing gums[52]. Other, describes a direct simultaneous determination of acetylsalicylic acid, caffeine, paracetamol and phenobarbital in tablets[53], or mixtures of paracetamol, caffeine and codeine phosphate[54]. The latest HPLC method was used to identificate mixtures of paracetamol, caffeine and ibuprofen in less than 10 minutes[55].

In some cases, if drugs are subject to abnormal conditions of temperature or pH, PCT can degradate into other products such as 4-aminophenol and acetic acid. This is a reaction that could also happen under the action of an enzyme. Not only 4-aminophenol could be present in the sample, but also 4-chloroacetanilide could be present as an impurity. A large variety of HPLC methods proposed take the presence of these contaminants into account, although they are time-consuming and the silica-based stationary phase can suffer from unwanted interactions[56,57].

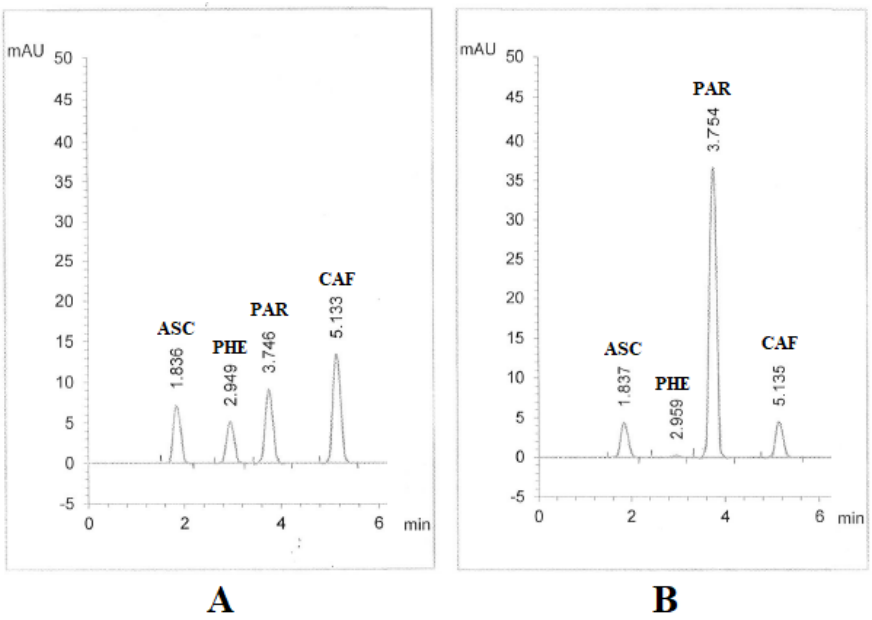

Figure 5. Examples of chromatograms[58] containing paracetamol and three other active ingredients commonly found in medicines: ascorbic acid (ASC), phenylephrine (PHE) and caffeine (CAF).

1.5. Selected method

The selected method among all the possible ones is reversed-phase HPLC with ultraviolet detection[58,59], because of the simplicity of the method, lack of a derivatization step in the sample preparation. It is a separation technique that allow us to isolate the signal related to PCT with good analytical parameters and better detection limits than the majority of spectrophotometric methods. The only drawback of this method is in comparison with electroanalytical techniques the lack of miniaturization of the process and the expensive instrumentation. Some of the interferent species present in the selected medicines are ascorbic acid, caffeine, chlorpheniramine, dextrometorphan and phenilephrine.

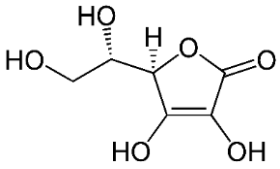

Figure 6. Structure of ascorbic acid.

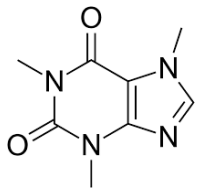

Figure 7. Structure of caffeine.

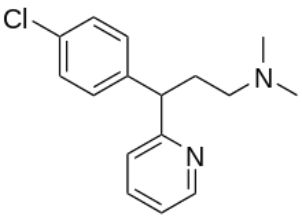

Figure 8. Structure of chlorpheniramine.

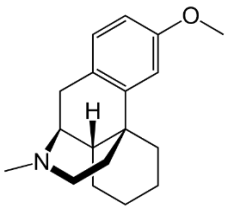

Figure 9. Structure of dextrometorphan.

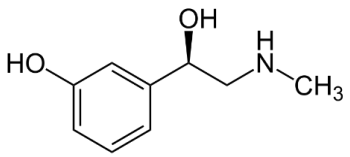

Figure 10. Structure of phenilephrine.

2. Objectives

The main objective of the present Bachelor thesis was the determination of paracetamol in pharmaceutical products. For this, an HPLC method in combination with an UV/Vis detector was optimized to ensure its reliable and interference-free determination. The method was then tested for the analysis of three commercially available products. In order to determine whether the designed method meet the expectations, analytical parameters such as resolution, selectivity, peak asymmetry and capacity factor of the peaks were taken into account.

3. Experimental Procedure

The experimental procedure to obtain analytical data from the samples consist of the injection of diluted aliquots of the medicines in an HPLC-system with a reversed-phase column and determine the concentration of paracetamol in various pharmaceutical products.

3.1. Instrumental

The HPLC employed is a Shimadzu modular system. It consists of a VP series FCV-10AL low pressure gradient valve, a VP series LC-10AT liquid chromatograph, a VP series SPD-10A UV-Vis detector and a CBM-20A communications bus module. The whole system is connected to the software LC Solution, which allows to control the mobile phase composition and displays the signal registered by the detector in real-time. The column employed is a Teknokroma Tracer Excel 120 ODS-A 3 µm 150×4.6 mm.

3.2. Reagents

Reagents for the mobile phase consists in HPLC grade acetonitrile, ROMIL-SpS gradient quality methanol and a Fischer acetic acid glacial buffer aqueous solution in milliQ water. The conjugated base is added in the form of PROBUS anhydrous sodium acetate. To prepare the standards Sigma 4-acetamido-phenol 99.0% (PCT) is used.

3.3. Chromatographic Conditions

In order to maximize the signal obtained by the detector, a prior UV spectrum of a PCT standard was performed.

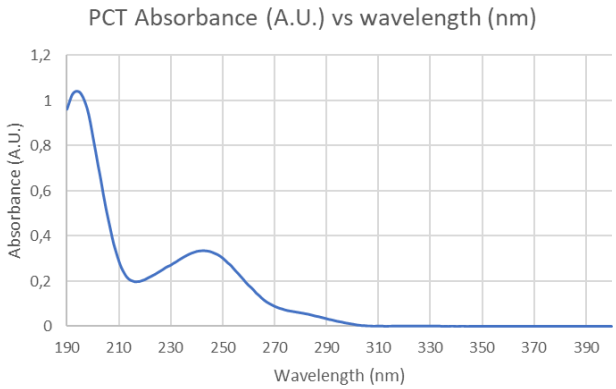

Figure 11. Recorded absorption spectrum of a paracetamol standard 5.05 ppm.

From the data of this spectrum, the detector wavelength is fixed at the maximum absorption: 242 nm.

The chromatograph is programmed to work with a mobile phase flow of 1 mL/min. The sample is injected manually using a Rheodyne valve. Injected volume is 20 µL.

The mobile phase is formed by a 0.05 M acetic acid buffer aqueous solution, acetonitrile and methanol. According to the reference paper[58], the composition of this mobile phase is 70% buffer, 20% acetonitrile and 10% methanol. The chromatograph used only allows the use of two mobile phase inlets at the same time, so in order to optimize the composition for the system in the inlet A the buffer was charged and a mixture 2:1 acetonitrile/methanol runs through the inlet B. After several tests, the optimal composition selected is 90% phase A and 10% phase B, which corresponds to a mixture 90% buffer, 6.7% acetonitrile and 3.3% methanol.

Table 2. Chromatographic conditions for the proposed method.

Parameters	Conditions
Column	Teknokroma Tracer Excel 120 ODS-A 3 µm 150×4.6 mm
Mobile Phase	0.05 M acetic acid buffer (pH 3.8), acetonitrile, methanol (90:6.7:3.3, v/v/v)
UV detection	242 nm
Flow rate	1 mL/min
Injected volume	20 µL
Temperature	Ambient

3.4. Standard solutions:

Dilute 10 mg of paracetamol reference standard in 100 mL of milliQ water. Take 4 aliquots of 0.1 mL, 0.5 mL, 1 mL and 2 mL and fill with milliQ water to a final volume of 10 mL.

3.5. Sample preparation:

The three analysed products come in a granulated form. According to the product information, Propalgina Plus (P) contains 500 mg of PCT, while Frenadol Complex (F) and Couldina Instant (C) contain 650 mg of PCT each. Other ingredients are present in smaller ammounts. Dilute each of the medicines in 250 mL of milliQ water. Medicine C is also effervescent, so degassing with helium is needed to remove all the CO_2 prior to the analysis. In order to eliminate insoluble particles, filtrate an approximated volume of 1 mL using a syringe and a 0.45 µm PVDF (polyvinylidene difluoride) filter.

4. Results and Discussion

4.1. Blank and calibration curves

In order to obtain the limit of detection (LOD) of the method, a blank is injected five times. From this data, the standard deviation of the blank is calculated.

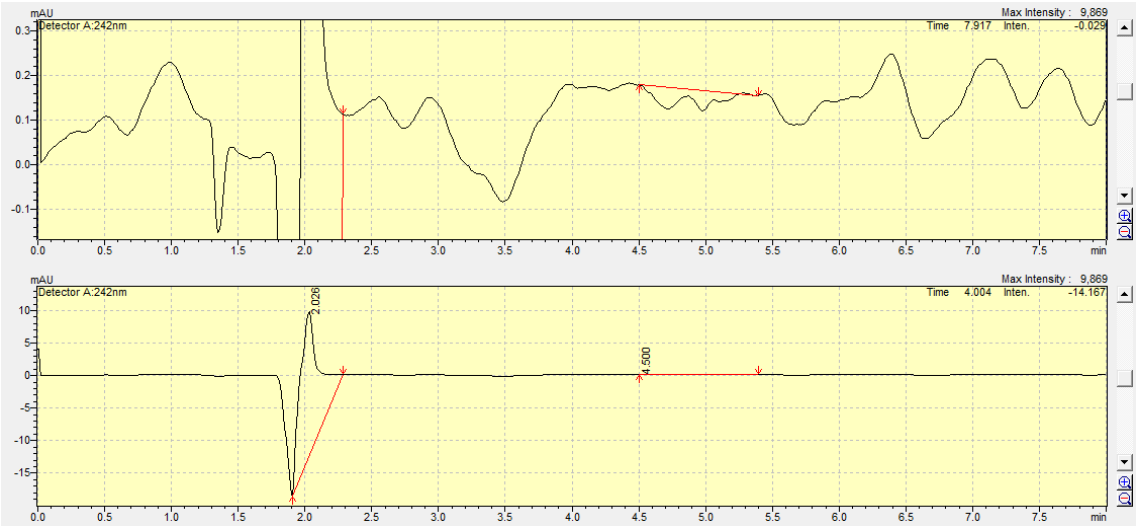

Figure 12. Chromatogram corresponding to the first blank measurement (miliQ water).

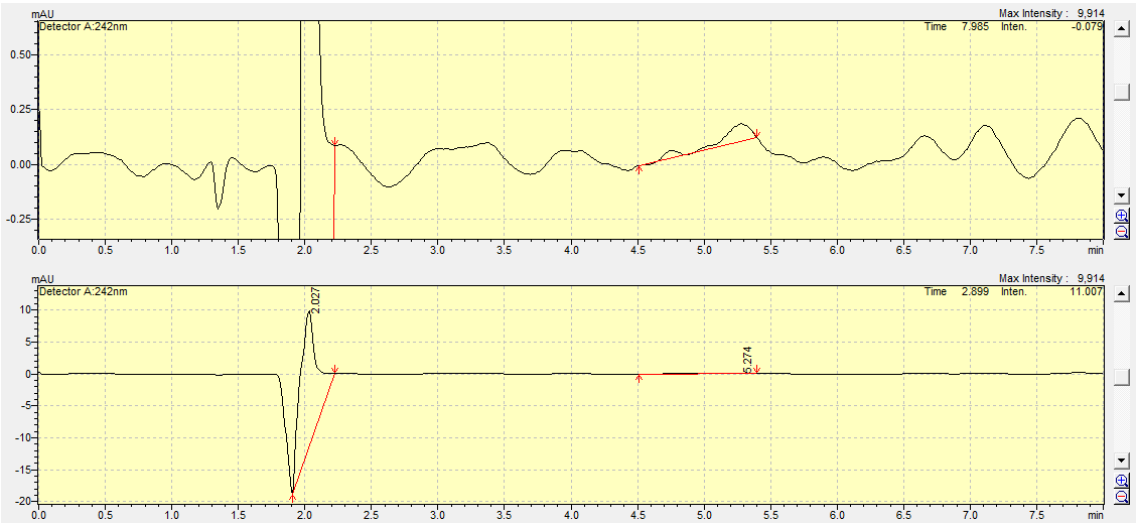

Figure 13. Chromatogram corresponding to the third blank measurement (miliQ water).

Due to high noise in the baseline the areas recorder in three out of five blank measurements are negative. However, this does not constitute a problem for the calculation of the limit of detection. The

areas are calculated in the range between 4.5 and 5.4 minutes, where the PCT elutes. The selected time range is justified with the chromatograms for the calibration.

Table 3. Areas of the blank (milliQ water) measured by quintupled.

Replicate	Blank Signal
1	-1127
2	-3215
3	1268
4	17
5	-1481

Mean signal of the blank = - 907.6

Standard Deviation = 1680

$$LOD = \frac{3 * \sigma_{blank}}{calibration\ slope} = 0.06\ ppm$$

$$LOQ = \frac{10 * \sigma_{blank}}{calibration\ slope} = 0.2\ ppm$$

The limit of detection (0.06 ppm) is well below the expected concentrations for the analysed samples, and also below the lowest calibration point. This means that the method is designed in a correct way, otherwise the lowest calibration point would not be detected.

The employed method of calibration is the external calibration method. To obtain a reliable result, each calibration point is measured by triplicate. Here is shown the chromatogram of the third replicate for the smallest concentration (1.01 ppm). The rest of the chromatograms obtained are available in the appendix (figures 25 to 36). As it can be observed, the shape of the peak is good. It resembles a Gaussian peak, with no observable peak tailing or broadening.

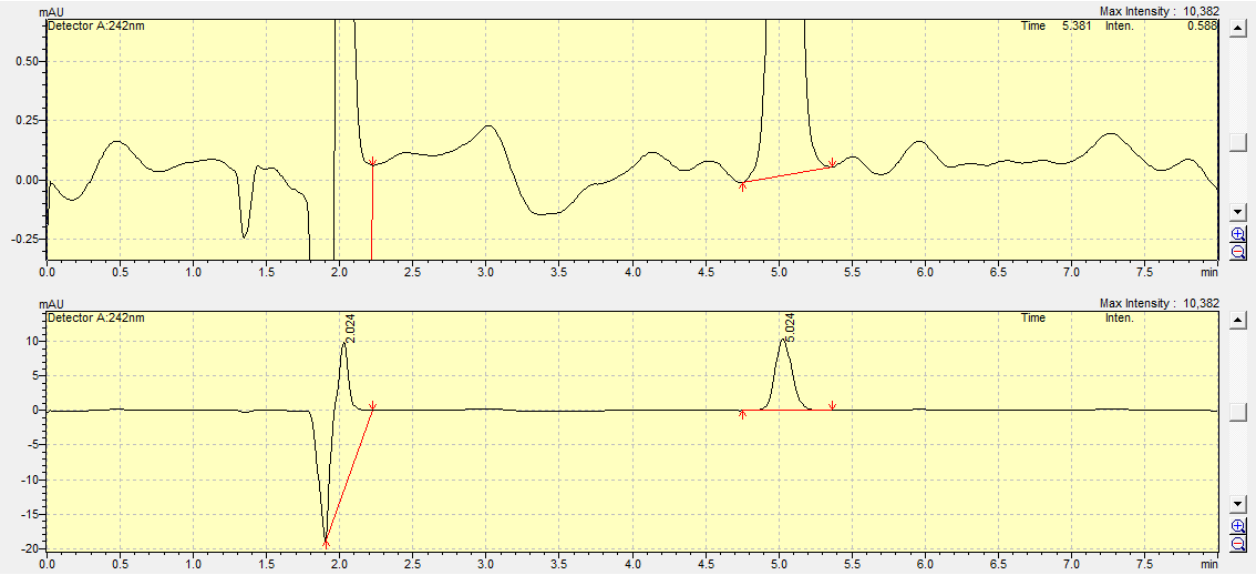

Figure 14. Chromatogram corresponding to the third measurement of a PCT standard of 1.01 ppm in miliQ water.

In the following table the areas and heights of the paracetamol peaks for each replicate are collected:

Table 4. Areas and heights of the PCT signal in the calibration chromatograms.

	Area			Height		
Concentration (ppm)	**Area 1**	**Area 2**	**Area 3**	**Height 1**	**Height 2**	**Height 3**
1.01	86923	83677	84115	10.498	10.154	10.382
5.05	446103	450584	447459	55.594	55.44	55.21
10.1	856909	855407	858956	106.243	106.799	106.261
20.2	1738632	1638759	1736488	213.454	200.392	210.367

Although the second measurement of the 20 ppm calibration standard is smaller than the first and third replicates, the difference only constitutes a difference of around 6% from the other two values. It does not result in a drastic change for the cuadratic expression and linearity to include or not this point. For these reasons all the points are considered correct, and no outliers are excluded in order to calculate the mean peak area and mean peak height.

Table 5. Mean peak areas and mean peak heights for the calibration performed by triplicate and its standard deviation. In the case of the blank a quintupled was performed.

Concentration (ppm)	Mean Peak Area	Area Std. Dev.	Mean Peak height	Height Std. Dev.
0	-907.6	1680	0.15	-
1.01	84905	1761	10.3	0.2
5.05	448049	2297	55.4	0.2
10.1	857091	1781	106.4	0.4
20.2	1737560	1516	212	3

With the data from this table, the two calibration lines are constructed. The first one using mean peak areas, and the second one with mean peak heights. In order to decide which one is better, compare both linearity coefficients. For the mean areas, R^2 has a value of 0.9998, and for the mean heights R^2 is also 0.9998. Linearity for both methods are very good (slightly higher for the calibration using peak heights). However, the reproducibility of the measurements in a different day is much more robust when using the area method. Performing a reference injection with the 10.1 ppm standard, results where just 2% lower than those in the calibration day. In the case of heights, results experimented a decrease of 24% with respect to the calibration value. For that reason, the mean peak area method is chosen. Both calibration curves with their corresponding cuadratic expressions are represented here:

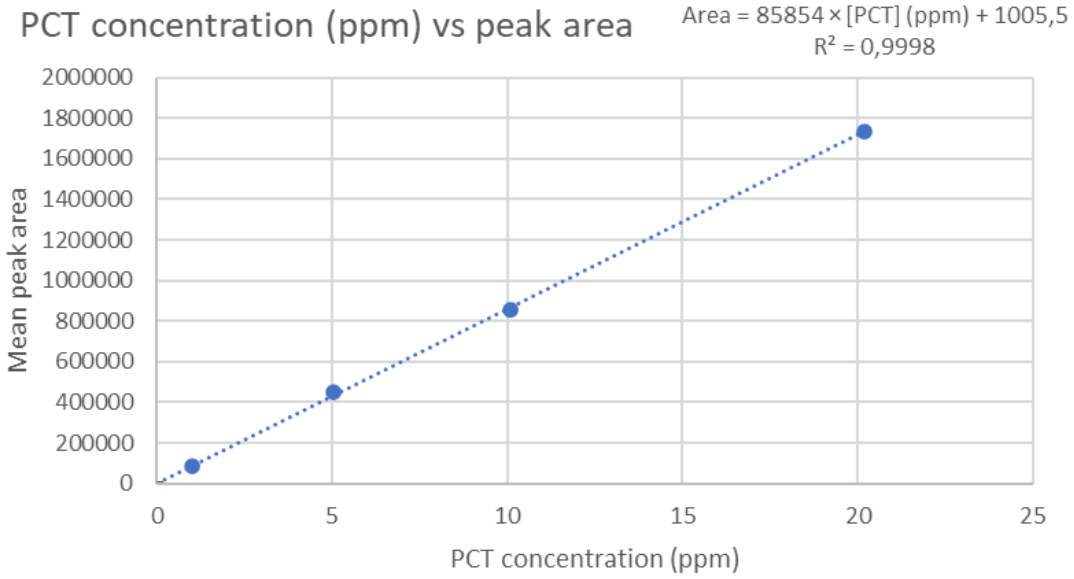

Figure 15. Calibration curve for PCT determination using mean peak areas.

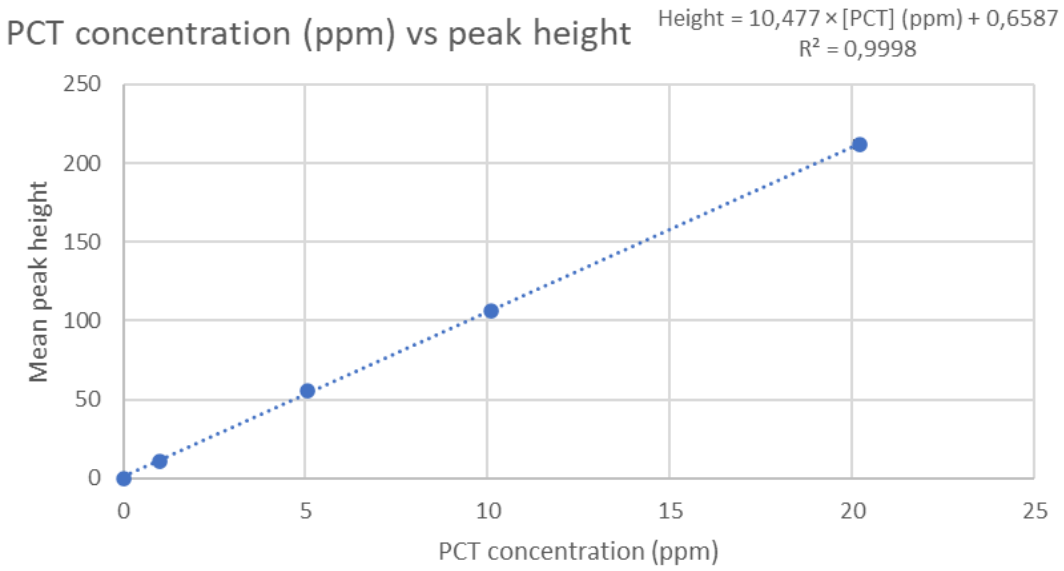

Figure 16. Calibration curve for PCT determination using mean peak heights.

4.2. Sample Injections

4.2.1. Couldina Instant (C)

The first medicine injected, Couldina Instant (C), has a declared content of 650 mg of paracetamol per dose. After dissolving and diluting the sample in milliQ water, the obtained chromatogram is shown below:

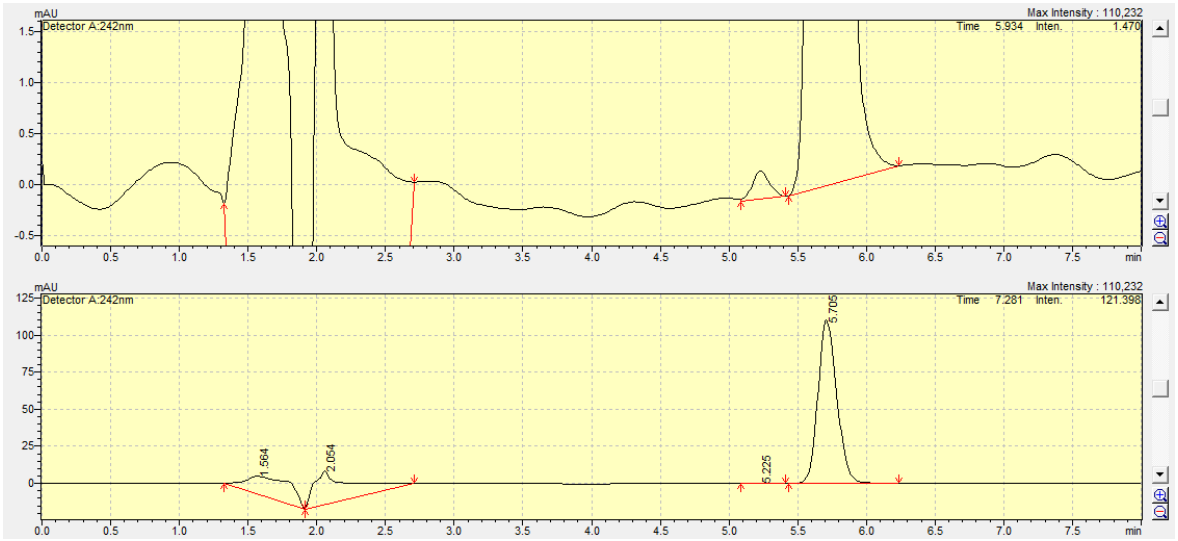

Figure 17. Chromatogram corresponding to the second replicate of medicine C injection.

Under the employed chromatographic conditions, the analyte elutes at 5.7 min. Retention factor for PCT is 2.8 calculated with equation 1, where t_0 is the dead time and t_R is the elution time of PCT. The retention factor is usually kept between 1 and 10 for a good separation.

$$k = \frac{t_R - t_0}{t_0} \quad (1)$$

Other UV active ingredients of this pharmaceutical product do not interfere the determination of PCT. The closest differentiable signal appears at 5.2 min, yielding a resolution for PCT between 1.7 and 2.1 after performing the measurement by triplicate. These values are sign of optimal conditions: complete separation between peaks and minimum analysis time.

Another important parameter indicating a correct design is the symmetry of the peaks. The paracetamol signals almost follow a gaussian shape, with very small peak tailing only observable in the zoomed-in image (tailing appears below 1% of the total peak height). The peak asymmetry factor is measured at 10% of the maximum height, so the value obtained is approximately 1.

The last factor analysed is the selectivity (α) of the system for PCT. This is calculated with equation 2, comparing retention factors of PCT and the previous peak. The selectivity is always higher than one, because the peak that elutes first is in the denominator while the most retained is in the numerator. Higher values indicate how good the apex of both peaks are separated. For medicine C, the selectivity is 1.13.

$$\alpha = \frac{k_2}{k_1} = \frac{t_{R2} - t_0}{t_{R1} - t_0} \quad (2)$$

The data obtained from this triplicate is grouped in a table and other analytical parameters such as the resolution (Rs), peak width at 50% of its height, number of plates (N) and the height equivalent to a theoretical plate (HETP). In order to obtain the PCT contained in the medicine, the dilution factor used to relate the obtained areas to the calibration is depicted here:

$$Medicine\ PCT\ (mg) = \frac{Area - intercept}{slope}\left(ppm(=)\frac{mg}{L}\right) * \frac{1\ L}{1000\ mL} * \frac{10\ mL}{0.05\ mL} * 250\ mL \quad (3)$$

Table 6. Signals obtained for PCT, measured content (mg), peak resolution (Rs) and the peak width at 50% of the height (min) in medicine C.

Couldina Instant (C)						
Replicate	**Area**	**PCT content (mg)**	**Rs**	**Width 50% (min)**	**N**	**HETP (mm)**
1	1056761	614.7	1.77	0.143	8818	0.0170
2	1056328	614.5	1.90	0.147	8344	0.0180
3	1063972	618.9	2.08	0.146	8459	0.0177

The determined average content of PCT in medicine C is 616 mg with a standard deviation of 3 mg.

The average number of theoretical plates (N) for the analysis of medicine C is 8500 ± 300. The height equivalent to a theoretical plate (HETP) in millimiters is on average 0.0176 ± 0.0005 mm. The number of plates is a measurement of peak dispersion on the column, reflecting its performance. A higher number of theoretical plates results in a better quality of separation. The equation 4 is used to calculate the theoretical plates, where t_R is the retention time of PCT and $w_{1/2}$ the width of the peak at half of the maximum height.

$$N = 5.54\left(\frac{t_R}{w_{1/2}}\right)^2 \quad (3)$$

This medicine contained in addition to 650 mg of PCT, 4 mg of chlorphenamine maleate and 10 mg of phenylephrine hydrochloride. Other compounds present catalogued as excipients are: Povidone K30 (E-1201), sodium docusate, citric acid anhydride (E-330), sodium hydrogencarbonate (E-500ii), sodium carbonate anhydride (E-500i), sodium saccharin (E-954) and lemon flavour.

Only four peaks appear consistently in the chromatograms. The first one is due to unretained compounds that absorb ultravoilet radiation, accompanied by the peak of the solvent (milliQ water). Another small peak is observed just before the PCT peak. Neither composition nor concentration of this compound can be known from this experiment. Later on by comparison with the other medicines, which contain different active ingredients, a discussion is made about the nature of these peaks.

4.2.2. Frenadol Complex (F)

The second medicine injected, Frenadol Complex (F), has also a declared content of 650 mg of PCT per dose. Preparation of the sample is done following the same method as before and the measurement is repeated three times. Below the chromatogram corresponding to the first replicate is shown:

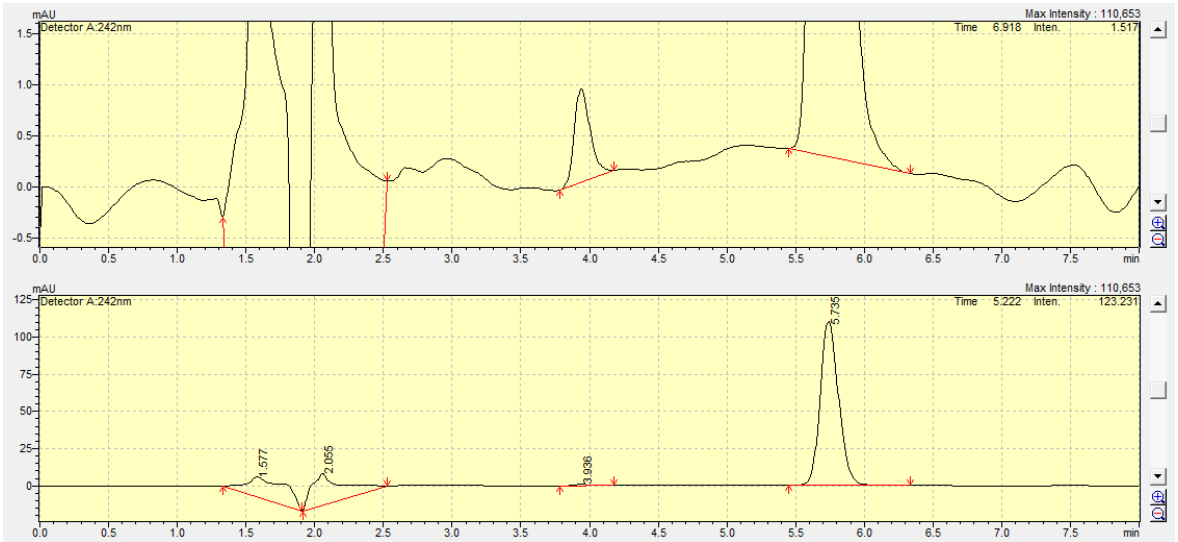

Figure 18. Chromatogram corresponding to the first replicate of medicine F injection.

As in the previous analysed pharmaceutical product, the peak we are interested in (PCT) elutes at 5.7 min, and no interferent signals are observed. The capacity factor remains at 2.8 like in the previous

case. The nearest signal due to an ingredient of this drug elutes at 3.9 min. For this reason both selectivity and resolution increase with respect to the first product. The resolution in this case oscillates between 6.2 and 7.7, while the selectivity (α) in this analysis for PCT is 1.77. The symmetry of the peak is as good as in the first sample, with minor tailing at heights below 1% of the maximum height.

The eluting strength could be increased in this particular case to work with smaller resolutions (still above 2). However, the conditions developed want to be applicable in a broad variety of medicines with PCT in its recipe. For that reason, the analysis is not further optimized in terms of mobile phase.

The data obtained from this triplicate is grouped in a table, containing also the associated PCT content in mg calculated employing the dilution factor (3):

Table 7. Signals obtained for PCT, measured content (mg), peak resolution (Rs) and the peak width at 50% of the height (min) in medicine F.

Frenadol Complex (F)						
Replicate	**Area**	**PCT content (mg)**	**Rs**	**Width 50% (min)**	**N Plates**	**HETP (mm)**
1	1029140	598.6	7.59	0.143	8911	0.0168
2	1028908	598.5	7.69	0.147	8432	0.0178
3	1028308	598.2	6.24	0.146	8548	0.0175

The average content of PCT in medicine F is 598.4 mg with a standard deviation of 0.3 mg.

The average number of theoretical plates for the analysis of medicine F is 8600 ± 300. The height equivalent to a theoretical plate (HETP) in millimiters is on average 0.0174 ± 0.0005 mm.

Other components present in this drug are: 20 mg of dextrometorphan hydrobromide, 4 mg of chlorphenamine maleate, 30 mg of caffeine citrate and 250 mg of ascorbic acid. In an unkown proportion as excipients the medicine contains also: sucrose, titanium dioxide (E-171), citric acid, polysorbate 80 (E-433), orange flavour (benzyl alcohol and sodium traces), quinoleine yellow (E-104) and sunset yellof FCF (E-110).

4.2.3. Propalgina Plus (P)

The last medicine, Propalgina Plus (P), has a declared content of 500 mg of PCT per dose. The sample preparation is the same depicted for the other pharmaceutical products. The base line noise increased for this particular medicine, but it does not constitute a problem for the PCT determination. The measurements were repeated seven times, due to huge differences between peak areas of the first

three replicates. There were also ghost signals in some of the chromatograms (figures 42 and 44) that did not appear more than once and could be related with a malfunction of the pumping system or the detector. After restarting the instrument a few times, only the signals due to compounds present in the medicine were observed and could be identified. An example of a good chromatogram is shown below:

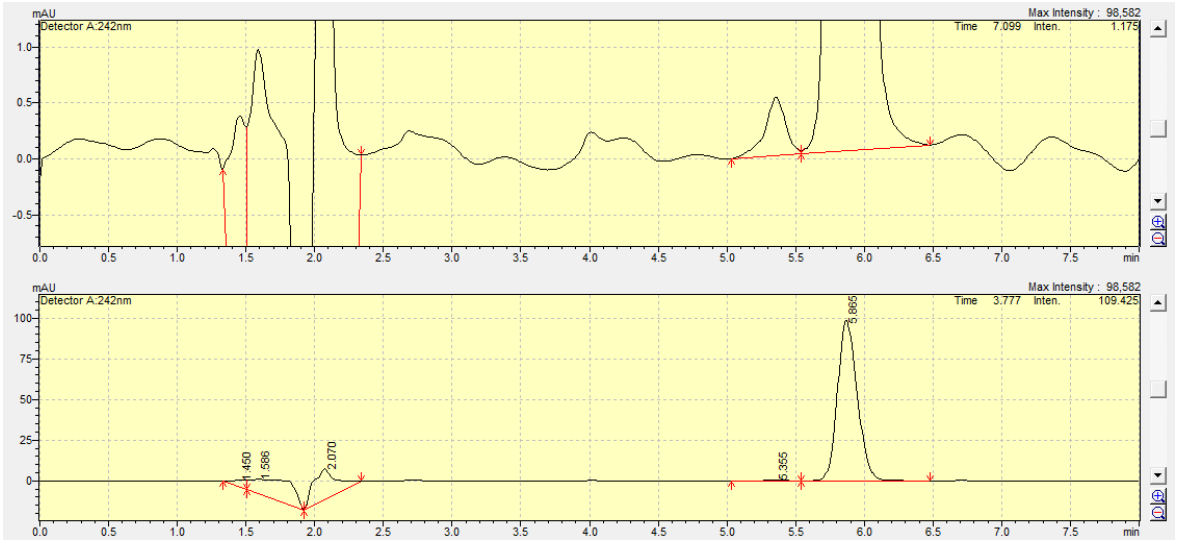

Figure 19. Chromatogram corresponding to the sixth replicate of medicine P injection.

The paracetamol peak has a retention time of 5.8 minutes, almost the same as the other two samples.

Another compound elutes right before the PCT, resulting in a peak at 5.3 min. Capacity factor remained at 2.8. Resolution of PCT in this goes from 0.57 in replicate 3, which is not representative because of previously mentioned anomalous peaks, to 2.46. The rest of the signals lie between 1.6 and 2.1, which are values similar to those for Couldina Instant. In the case of selectivity, calculated with equation 2, the result obtained is 1.13. Peak shape is gaussian aswell, with slight peak tailing at heights not large enough to be taken into account.

The following table contains all the analytical parameters obtained for Propalgina Plus, such as the peak area, the related PCT content to that signal, resolution, width at half maximum height, number of theoretical plates and the height equivalent to a theoretical plate:

Table 8. Signals obtained for PCT, measured content (mg), peak resolution (Rs) and the peak width at 50% of the height (min) in medicine P.

Propalgina Plus (P)						
Replicate	**Area**	**PCT content (mg)**	**Rs**	**Width 50% (min)**	**N Plates**	**HETP (mm)**
1	877393	510.2	1.77	0.147	8819	0.0170
2	869813	505.8	2.04	0.146	8940	0.0168
3	959683	558.1	0.57	0.142	9451	0.0159
4	1010642	587.9	2.46	0.149	8584	0.0175
5	982660	571.5	1.98	0.148	8700	0.0172
6	988160	574.7	1.90	0.154	8035	0.0187
7	965556	561.6	1.67	0.147	8819	0.0170

The analysis gave very different results in the case of this last medicine. The measured content in average is 550 mg with a standard deviation of 40 mg.

The average number of theoretical plates for the analysis of medicine P is 8800 ± 500. The height equivalent to a theoretical plate in millimiters is on average 0.0172 ± 0.0008 mm.

Active ingredients present in the drug appart from PCT are: 10 mg of dextrometorphan hydrobromide, 2 mg of chlorphenamine maleate, 7.5 mg of phenylephrine hydrochloride and 200 mg of ascorbic acid. In addition, other excipients are: Sodium saccharin (E-954), sodium cyclamate (E-952), citric acid anhydride (E-330), mannitol (E-421), sucrose, sodium edetate, anhydrous colloidal silica, povidone K30 and cola flavouring colorant.

4.3. Discussion of results

In the calibration, the PCT signal had a retention time of around 5.03 minutes, while the PCT in the medicines analysed appear between 5.6 and 5.8 minutes. The phenomena of peak shifting could be caused by different reasons. If the shift is observed in every peak, the most probable explanation is a variation in the instrument flow rate. However, the dead time peak they all share in common does not suffer any shift, so the varying flow rate hypothesis is discarded. If only some peaks are affected by this shifting, several things could be happening: Sample matrix could be interfering with the elution of PCT, a difference in pH between sample and mobile phase, the column has a build-up of sample matrix or that the column has reached the end of its lifetime. The most probable effect among these is the build-up of sample matrix in the column, because this is often accompanied by noisy baseline and/or

ghost peaks. In order to correct this a procedure of column cleaning and restoration should be followed.

To give an approximation of the compounds present in the pharmaceutical products without using reference standards of each of them, a comparative table can be employed to at least discard some posibilities:

Table 9. Comparative of the active ingredients present in each pharmaceutical formulation.

Active ingredient	Couldina Instant (C)	Frenadol Complex (F)	Propalgina Plus (P)
Chlorphenamine	Present	Present	Present
Phenylephrine	Present	Not present	Present
Dextrometorphan	Not present	Present	Present
Caffeine	Not present	Present	Not Present
Ascorbic acid	Not present	Present	Present

The peak observed at 5.3 min in medicines C and P must be due to a common ingredient present in both drugs. One of the compounds that could be the cause of that signal is phenylephrine hydrochloride, present only in medicines C and P. Chlorphenamine maleate is part of the three analysed drugs and no common peaks are observed in the three chromatograms apart from the PCT, solvents and unretained compounds, so chlorphenamine maleate must be part of those unretained compounds. In a similar way, dextrometorphan hydrobromide is present in medicines F and P, but no unknown peaks show a relation in the chromatograms of each drug. Peak observed at time 3.9 in medicine F might be due to the caffeine in the form of caffeine citrate contained in this drug, as it is an exclusive ingredient not present in the other two medicines.

The calculated contents of paracetamol are gathered in the following table, where they are compared to the declared content by the manufacturer:

Table 10. Declared contents and determinedl contents of paracetamol in mg in the analysed medicines.

Medicine	Declared PCT content (mg)	Calculated PCT content (mg)
Couldina Instant	650	616 ± 3
Frenadol Complex	650	598.4 ± 0.3
Propalgina Plus	500	550 ± 40

Both values for Couldina Instant and Frenadol Complex are below the declared paracetamol content. Possible reasons to justify the lower measured content is that some of the PCT is retained in the filter employed to prepare the samples. In order to check this possibility, the analysis should have been repeated without filtering and note if any substantial changes are recorded for the signal of PCT. In any case, the replicates had a very small deviation, with errors of 0.5% for Couldina Instant and 0.05% for Frenadol Complex, which is representative of a good test design that works very well isolating and measuring peaks of paracetamol in samples with complex matrices. The second hypothesis, reinforced by the quality of the results is that the products in fact contain less paracetamol than the declared amount (around a 5% for Couldina Instant and an 8% for Frenadol Complex).

The results for Propalgina Plus are less precise, with a deviation of 40 miligrams (accounting for an error of 7.3%). The obtained result also shows that the paracetamol contained in each pharmaceutical envelope is higher than the declared by 50 miligrams, a quite high quantity that gains importance in the scenario of a regular intake of 3 doses per day. The chromatograms do not show any clue of peak overlapping that could be causing this increase in the PCT signal.

Resolution of all of the analyses are above 1.5, which combined with an asymmetry factor of the PCT peak approximately equal to 1, capacity factor between 1 and 10 and a selectivity ranging from 1.13 in the analyses of Couldina Instant and Propalgina Plus to 1.77 in the Frenadol Complex analysis, account for a well designed procedure that can be considered close to the optimal conditions.

Number of theoretical plates is above 8000, a reasonably good number to obtain nice chromatograms with narrow gaussian peaks.

5. Conclusions

The fact that several signals appear in the different chromatograms performed indicate that either a separation technique or a highly selective analysis is needed. The selected medicines contain several active ingredients and excipients making harder the selection of a selective technique that works for all the samples. In addition, high-performance liquid chromatography combines the separation of compounds with a sensitive analysis (LOD < 0.1 ppm).

Analysis run time for all the samples were under 7 minutes, which is a quite fast time that allows to run several analyses within short time. Linearity is observed over a concentration range of 1-20 ppm for PCT.

The results obtained in the case of Couldina Instant and Frenadol Complex are really good, with very small deviations and close to the declared values by the Pharma. Both calculated concentrations are slightly lower than the declared, which might be an effect of the filtration step. In order to check this, the filtration should be avoided in further analysis, to find out if other interferent signals appear, or if the PCT concentration is higher than in the case of including a filtering step.

The concentration of paracetamol in Propalgina Plus is abnormally high with respect to the reference value. This could be due to the overlapping of other UV active species eluting at the same time than paracetamol. To ensure that no compound elutes at the same time than paracetamol, the analysis time should be increased employing a more polar mobile phase. If the concentration values remain the same after further tests, we can conclude that they are correct, and this product in fact contains more paracetamol than the declared values. This is important because it can result in overdoses and adverse effects, specially for kids, elders or people with hepatic conditions.

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7. Appendix

7.1. Ultraviolet Spectra

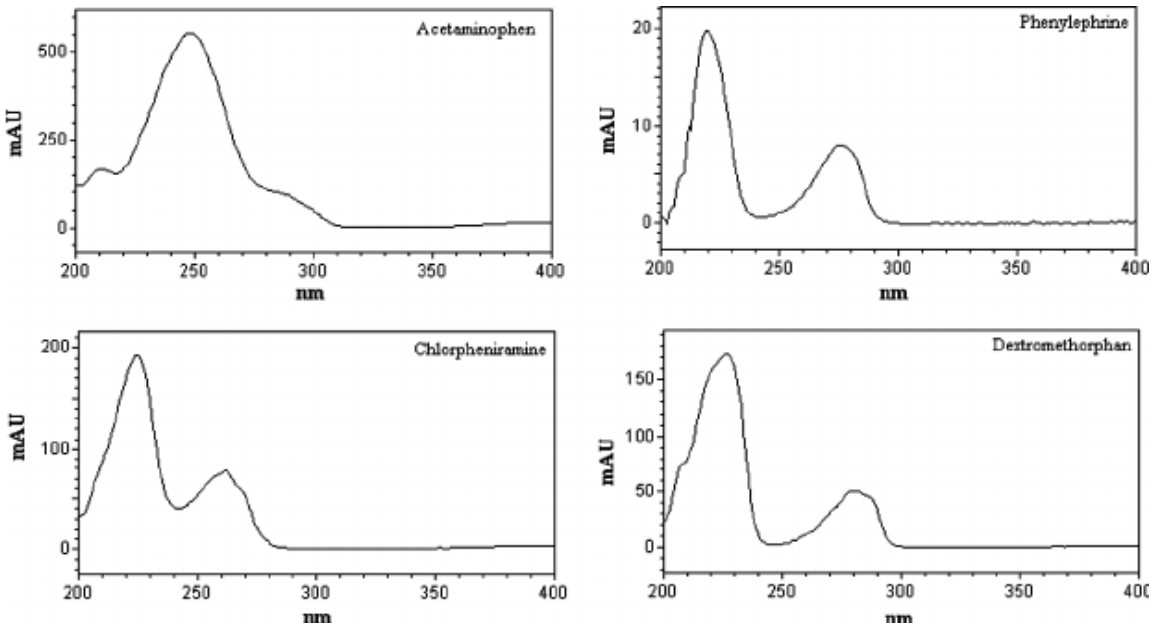

Figure 20. UV absorption spectra of acetaminophen (paracetamol), phenylephrine, chlorpheniramine and dextrometorphan[60].

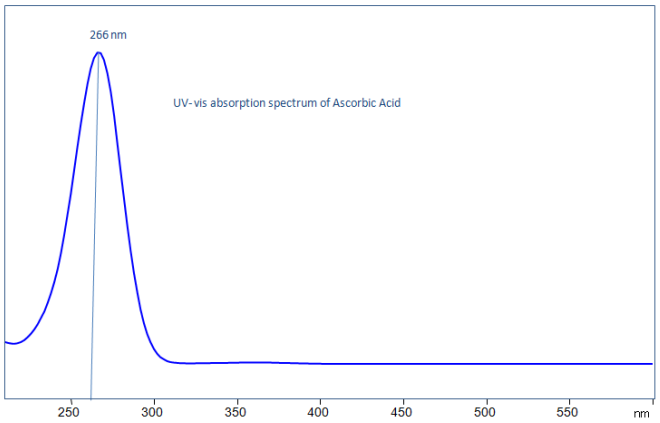

Figure 21. UV-Vis absorption spectra of ascorbic acid.

7.2. Chromatograms

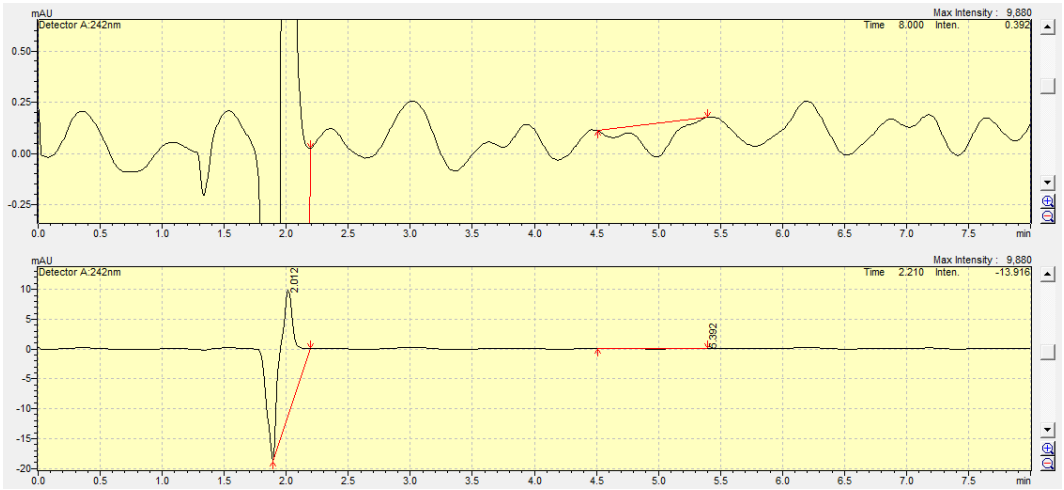

Figure 22. Chromatogram corresponding to the second blank measurement (miliQ water).

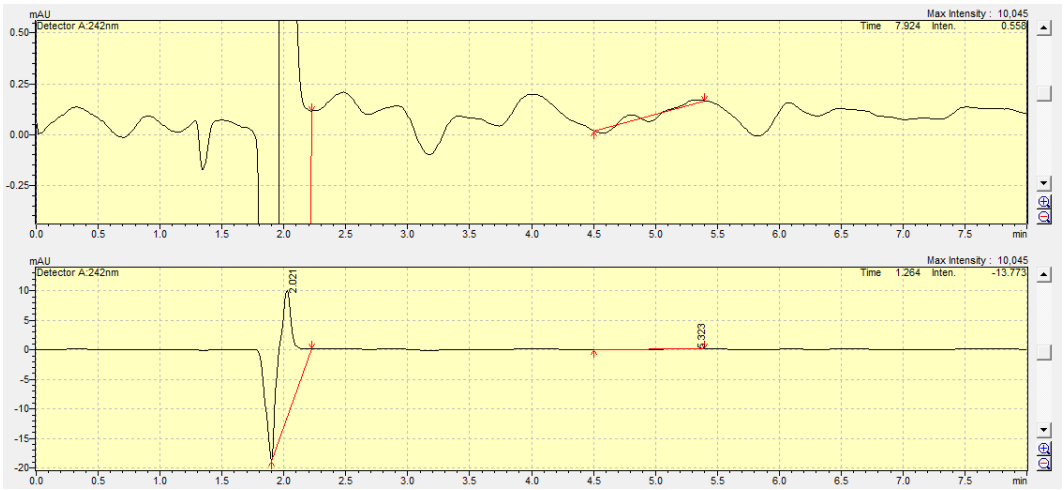

Figure 23. Chromatogram corresponding to the fourth blank measurement (miliQ water).

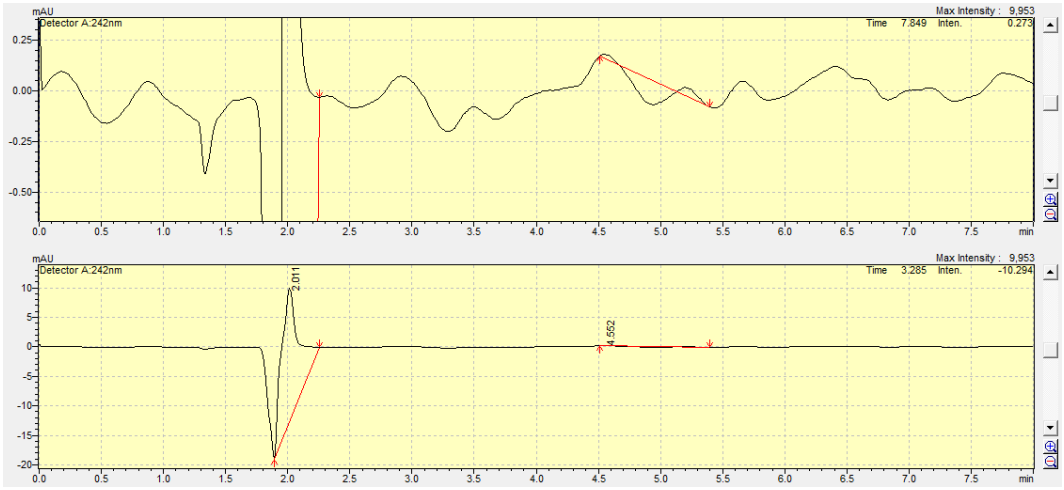

Figure 24. Chromatogram corresponding to the fifth blank measurement (miliQ water).

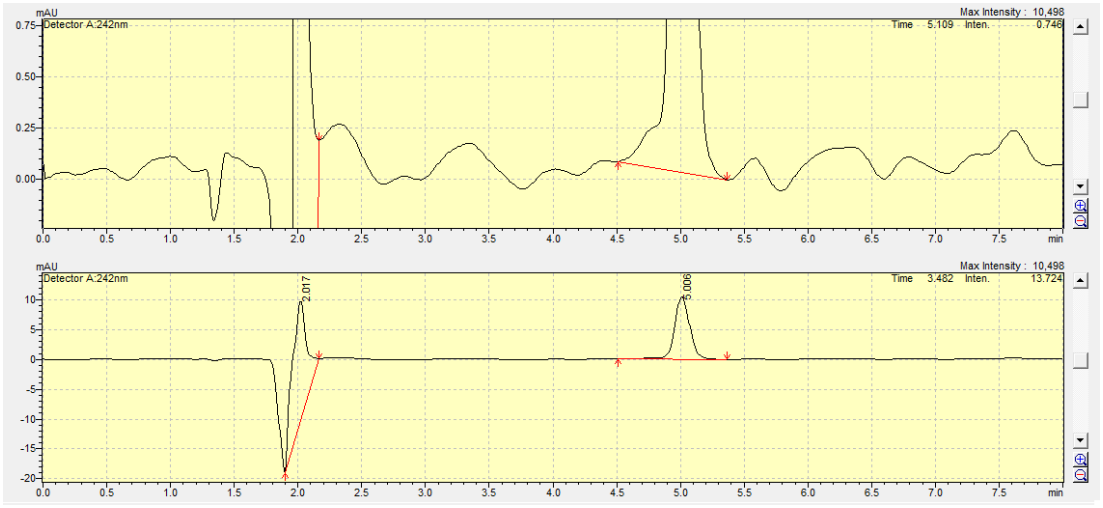

Figure 25. Chromatogram corresponding to the first measurement of a PCT standard of 1.01 ppm in miliQ water.

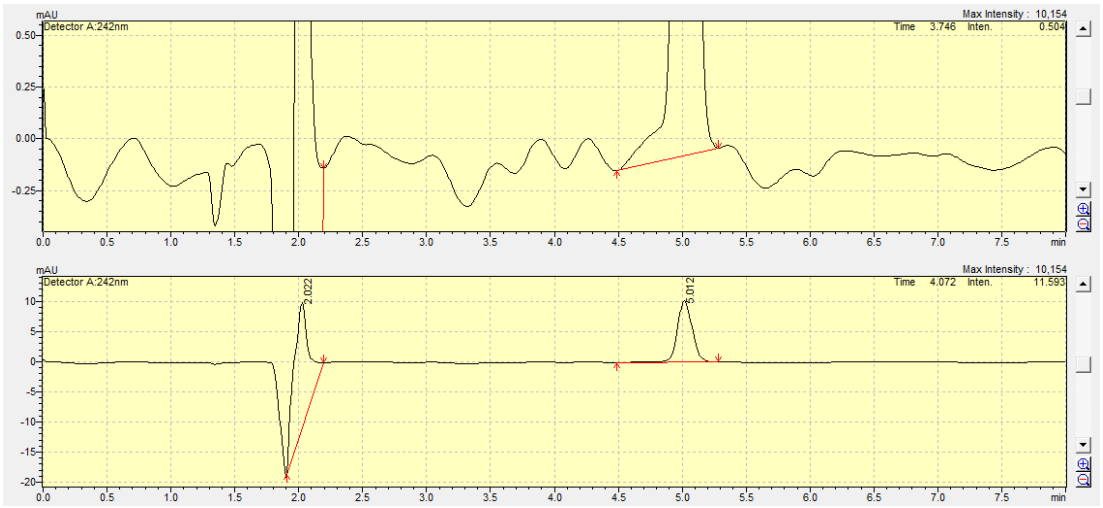

Figure 26. Chromatogram corresponding to the second measurement of a PCT standard of 1.01 ppm in miliQ water.

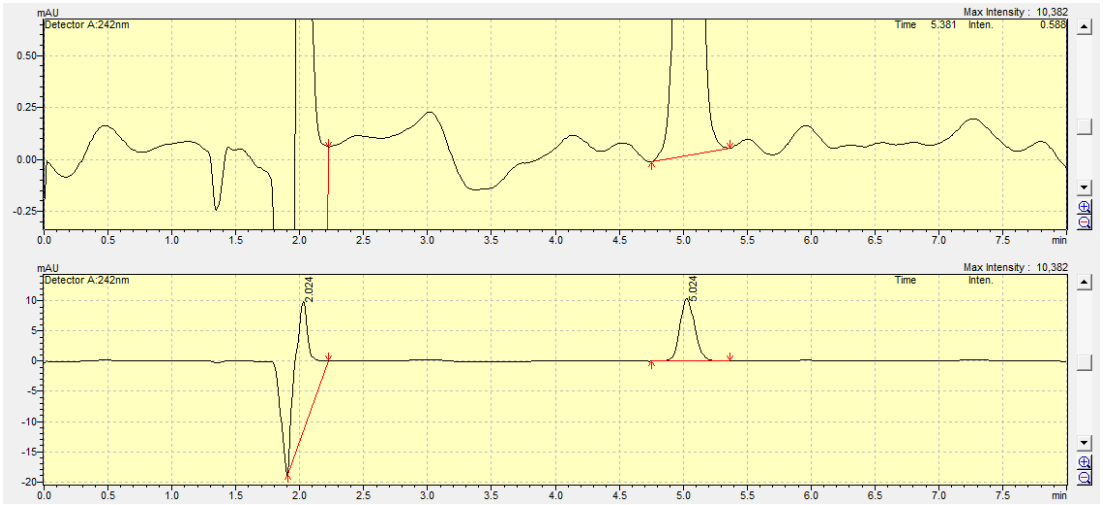

Figure 27. Chromatogram corresponding to the third measurement of a PCT standard of 1.01 ppm in miliQ water.

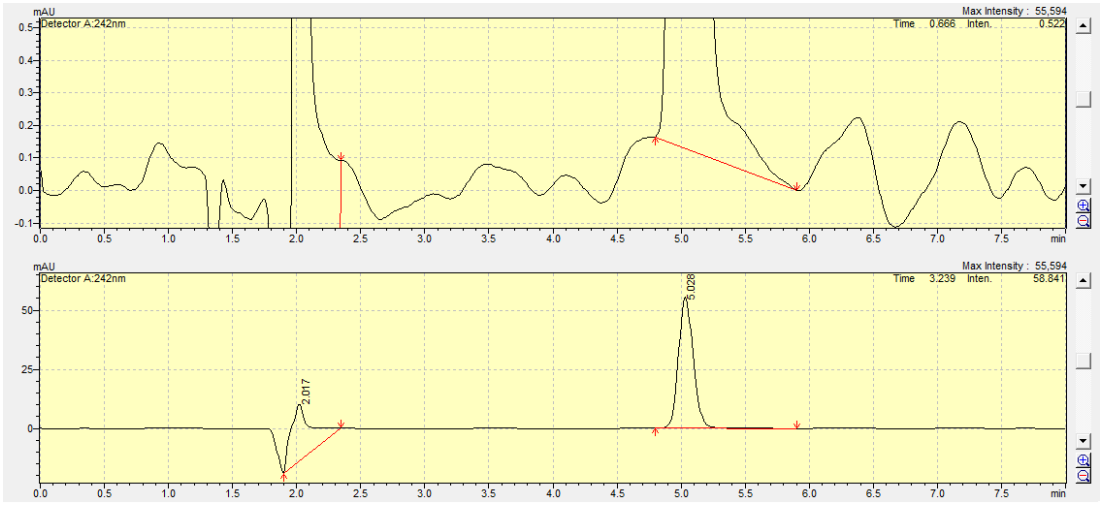

Figure 28. Chromatogram corresponding to the first measurement of a PCT standard of 5.05 ppm in miliQ water.

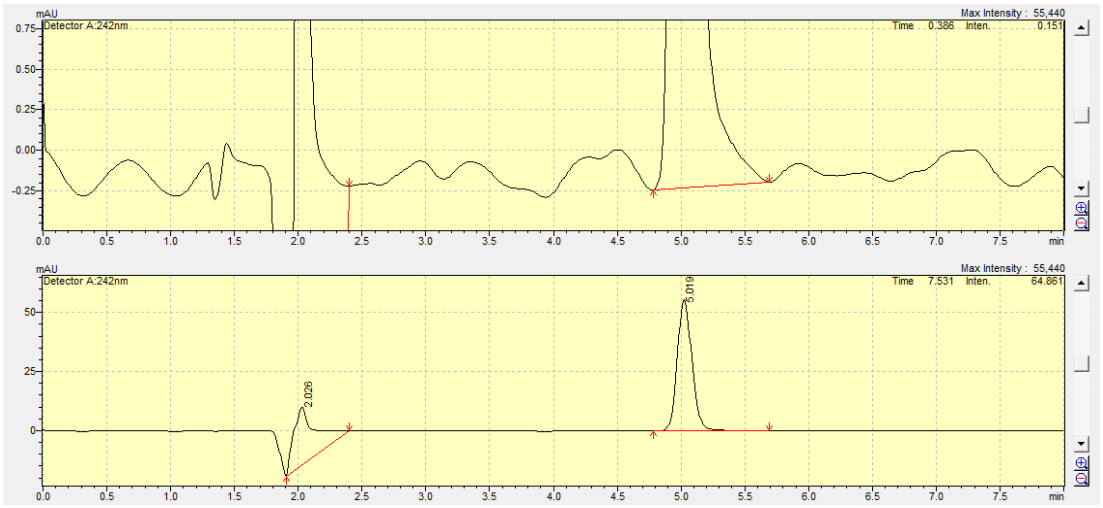

Figure 29. Chromatogram corresponding to the second measurement of a PCT standard of 5.05 ppm in miliQ water.

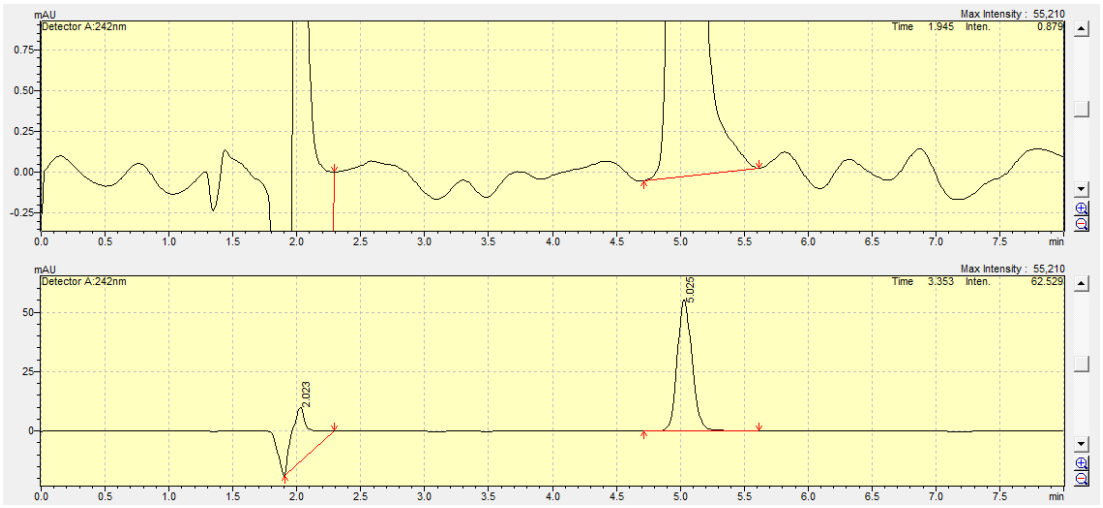

Figure 30. Chromatogram corresponding to the third measurement of a PCT standard of 5.05 ppm in miliQ water.

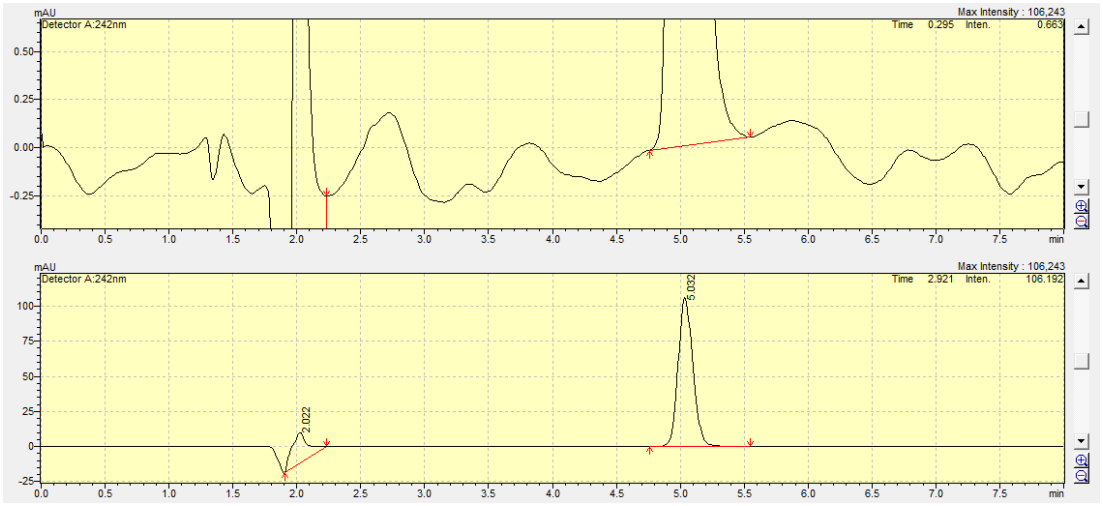

Figure 31. Chromatogram corresponding to the first measurement of a PCT standard of 10.1 ppm in miliQ water.

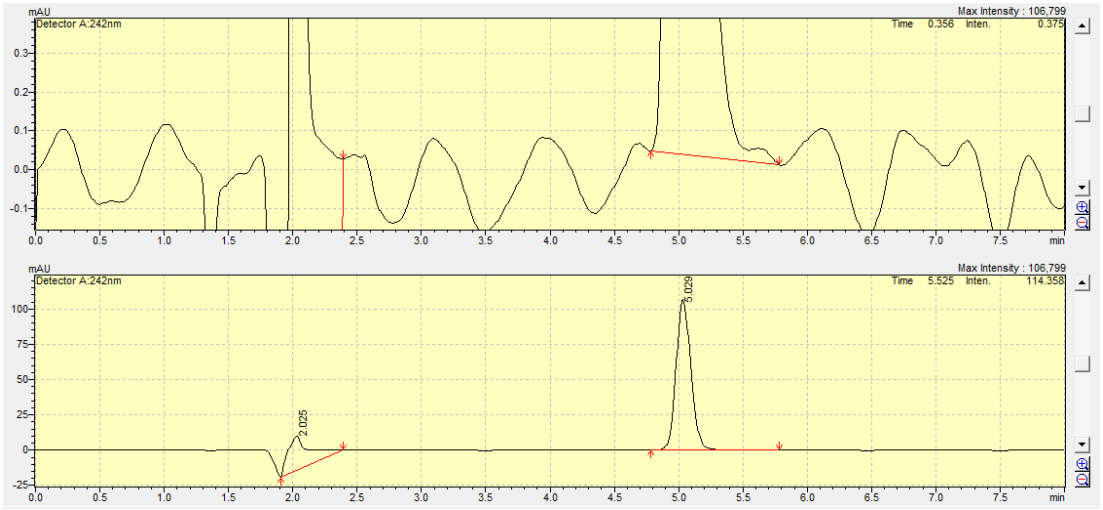

Figure 32. Chromatogram corresponding to the second measurement of a PCT standard of 10.1 ppm in miliQ water.

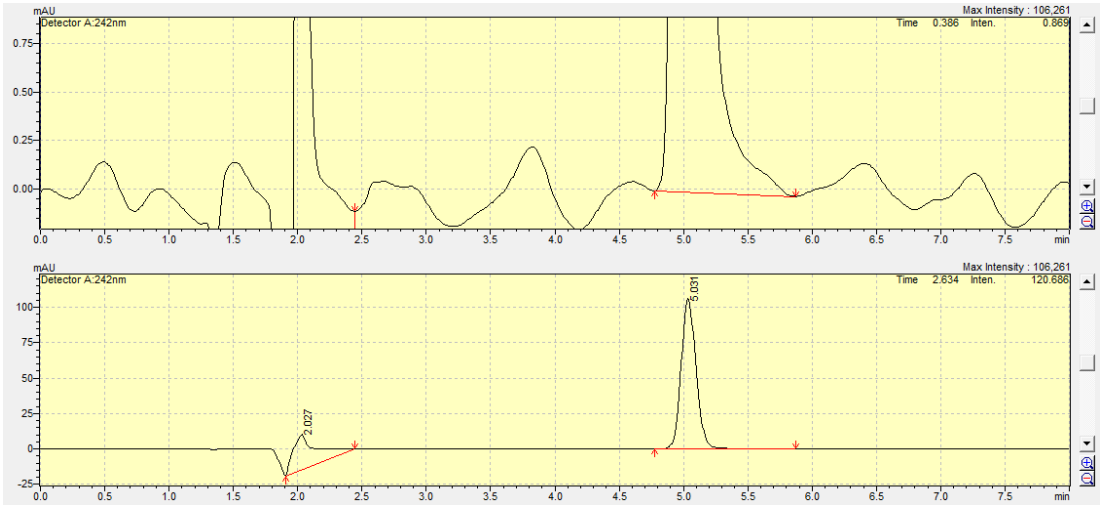

Figure 33.Chromatogram corresponding to the third measurement of a PCT standard of 10.1 ppm in miliQ water.

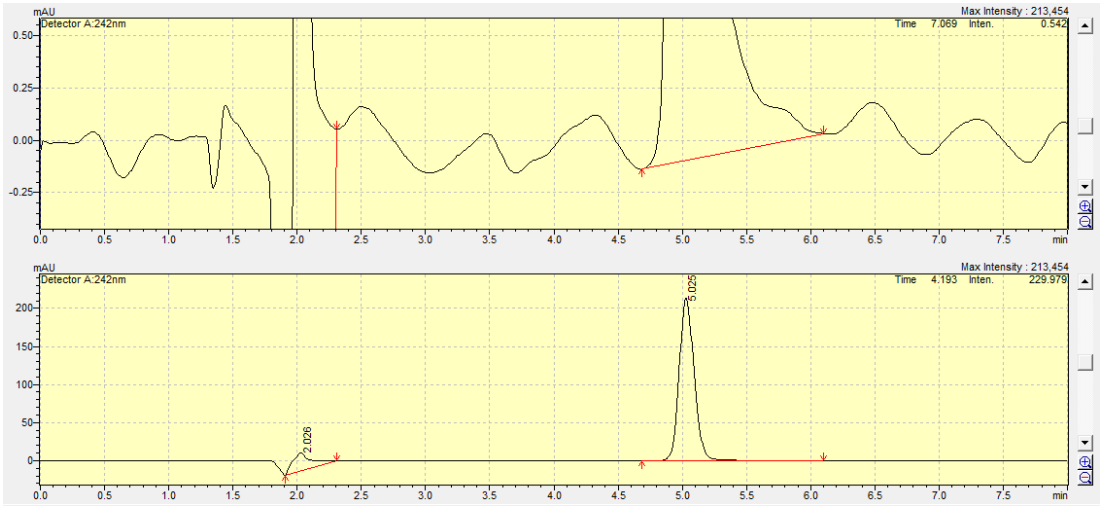

Figure 34. Chromatogram corresponding to the first measurement of a PCT standard of 20.2 ppm in miliQ water.

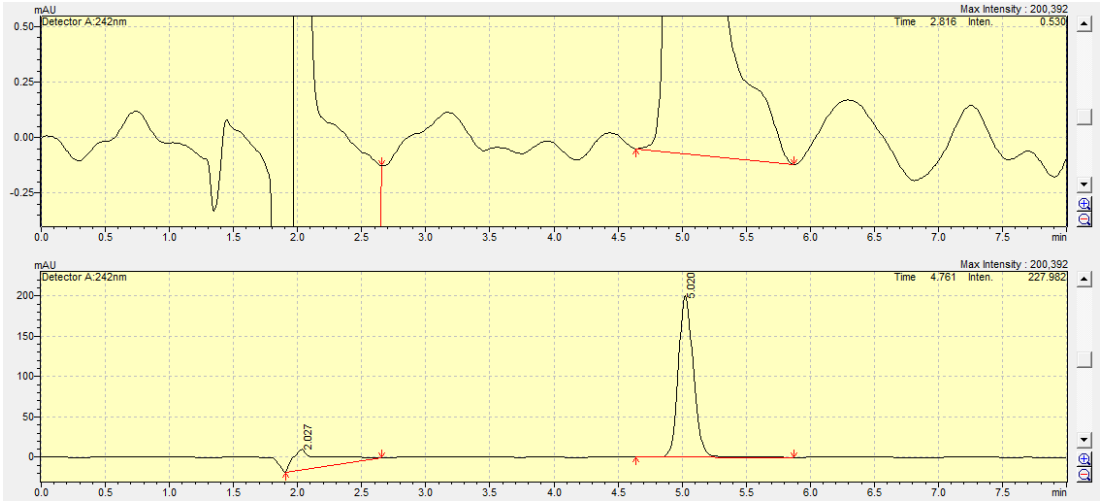

Figure 35. Chromatogram corresponding to the second measurement of a PCT standard of 20.2 ppm in miliQ water.

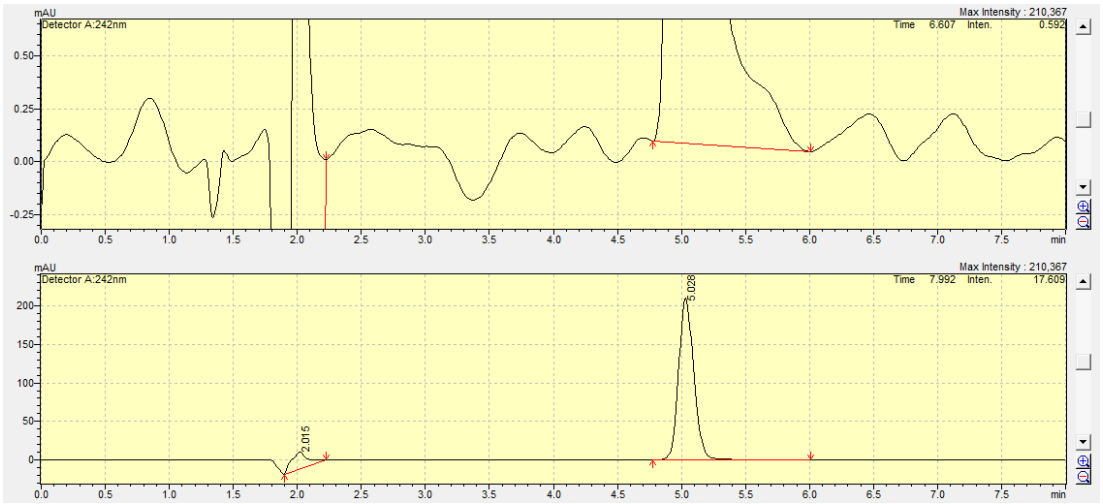

Figure 36. Chromatogram corresponding to the third measurement of a PCT standard of 20.2 ppm in miliQ water.

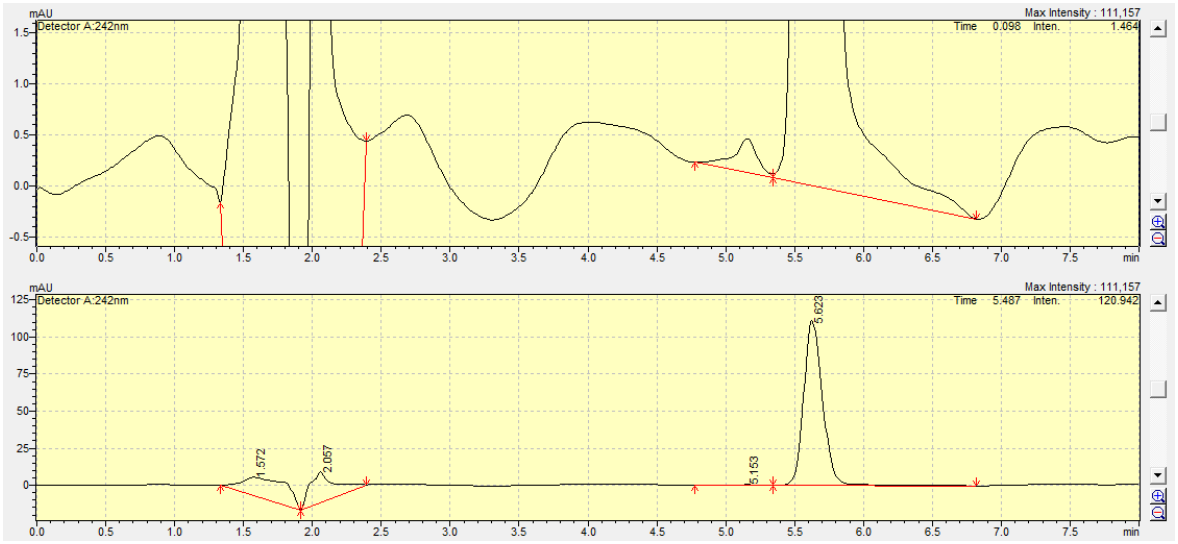

Figure 37. Chromatogram corresponding to the first replicate of the medicine C injection.

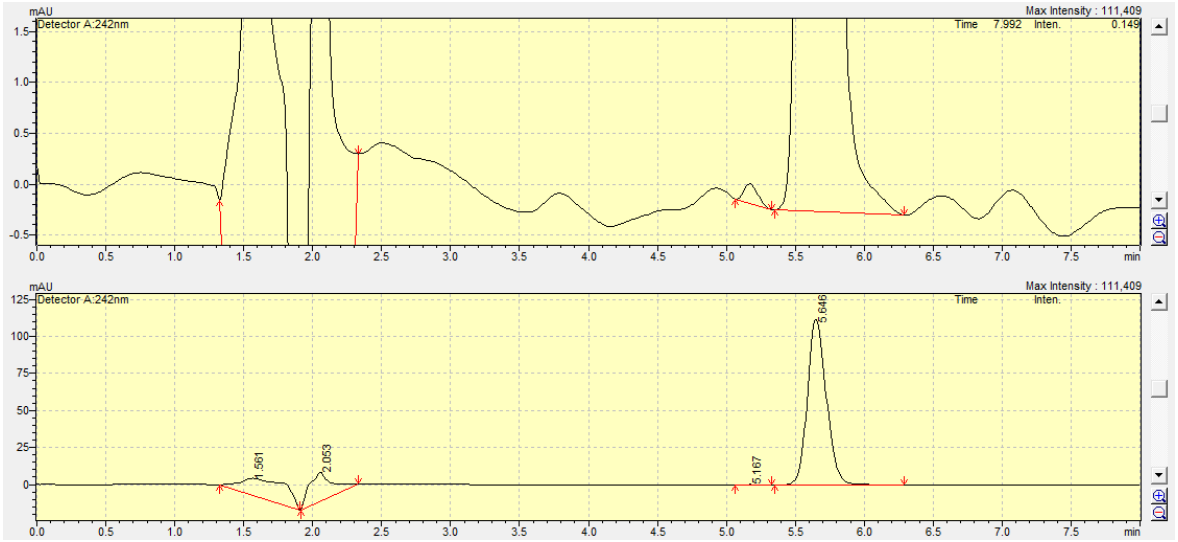

Figure 38. Chromatogram corresponding to the third replicate of the medicine C injection.

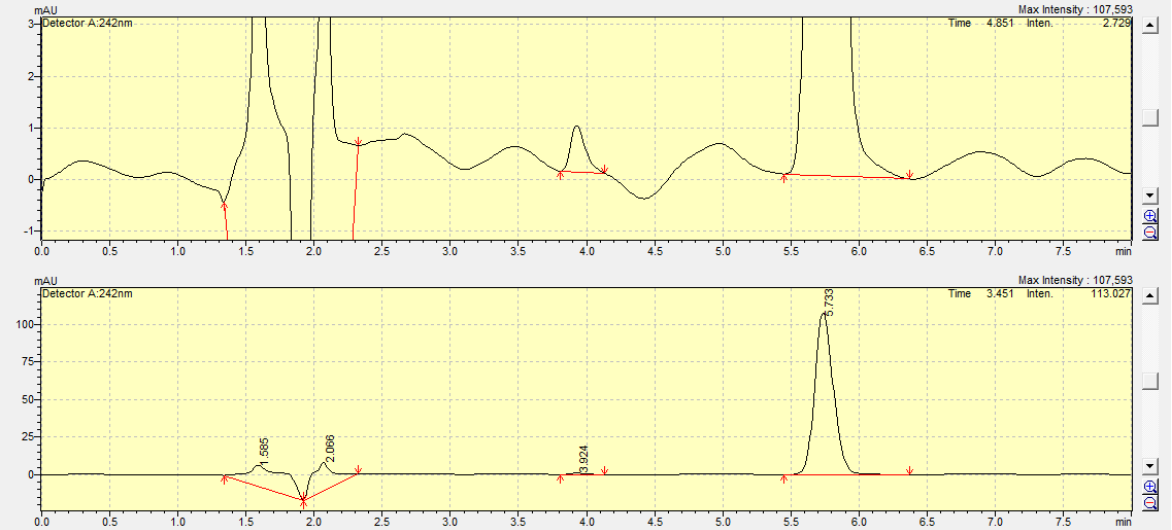

Figure 39. Chromatogram corresponding to the second replicate of the medicine F injection.

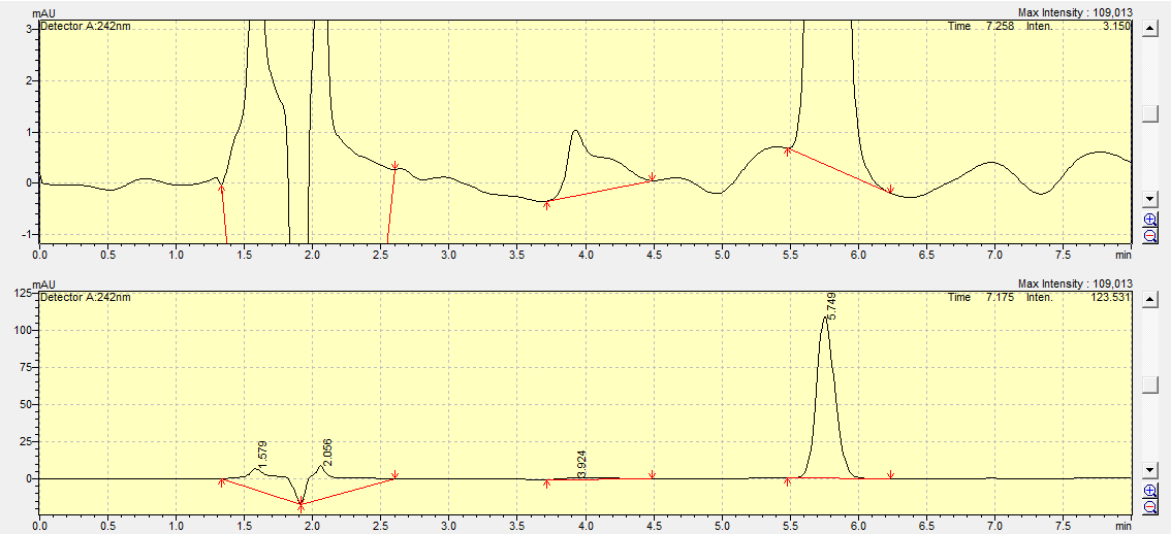

Figure 40. Chromatogram corresponding to the third replicate of the medicine F injection.

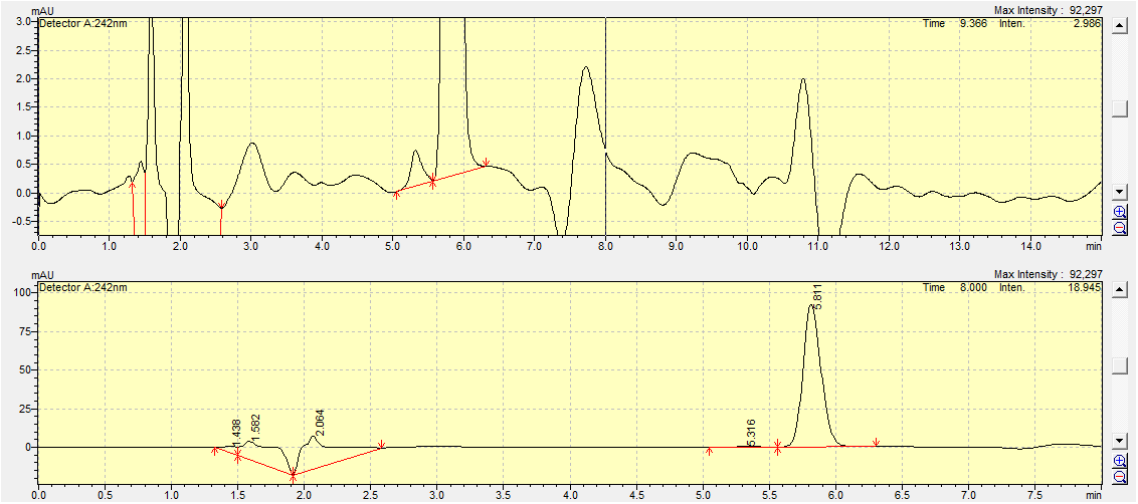

Figure 41. Chromatogram corresponding to the first replicate of the medicine P injection.

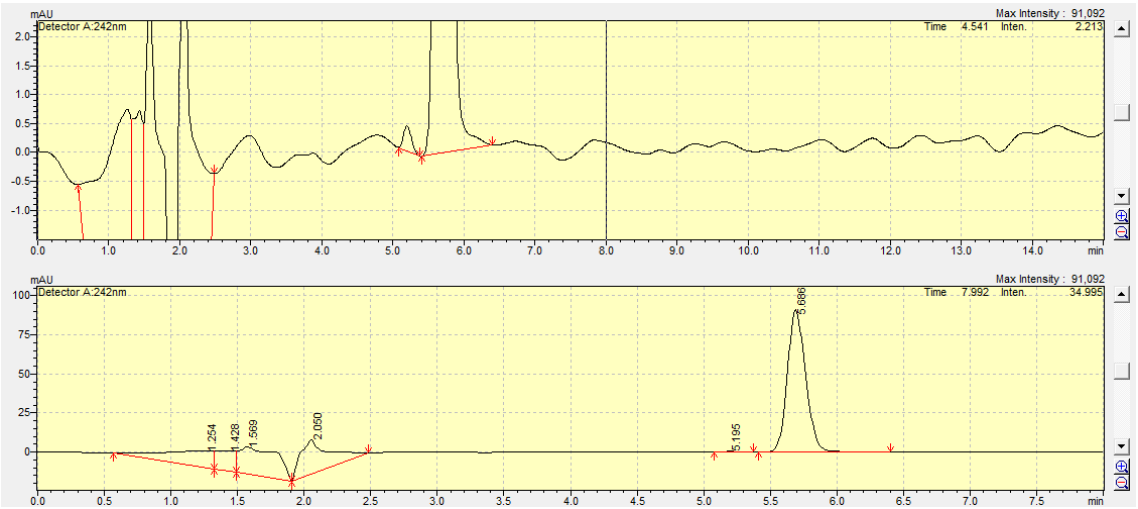

Figure 42. Chromatogram corresponding to the second replicate of the medicine P injection.

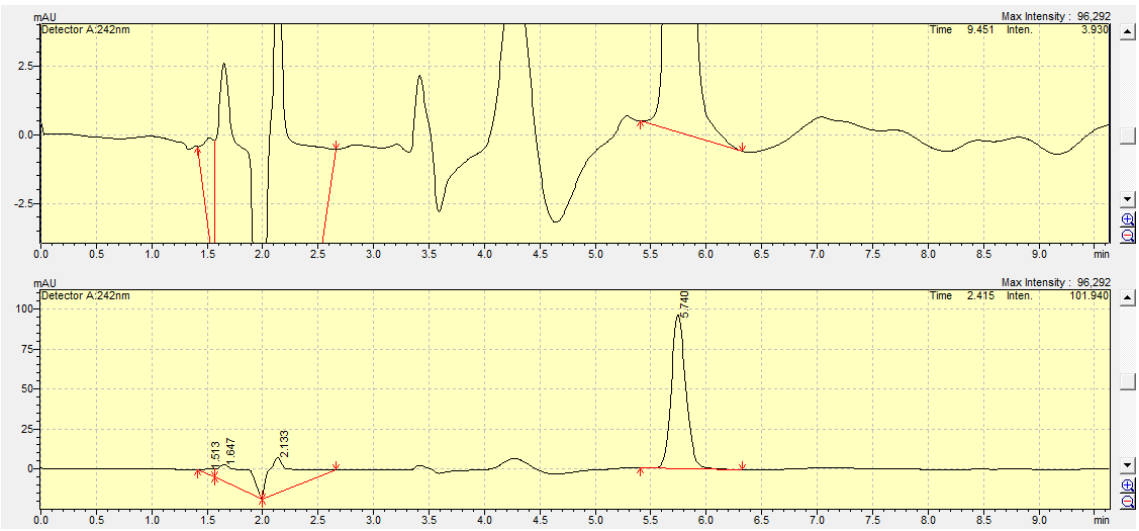

Figure 43. Chromatogram corresponding to the third replicate of the medicine P injection.

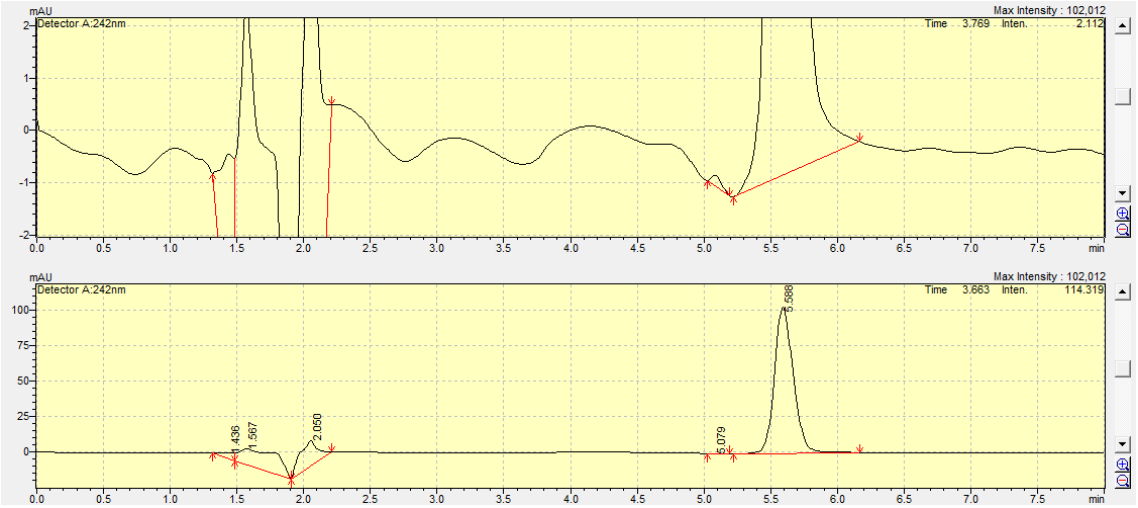

Figure 44. Chromatogram corresponding to the fourth replicate of the medicine P injection.

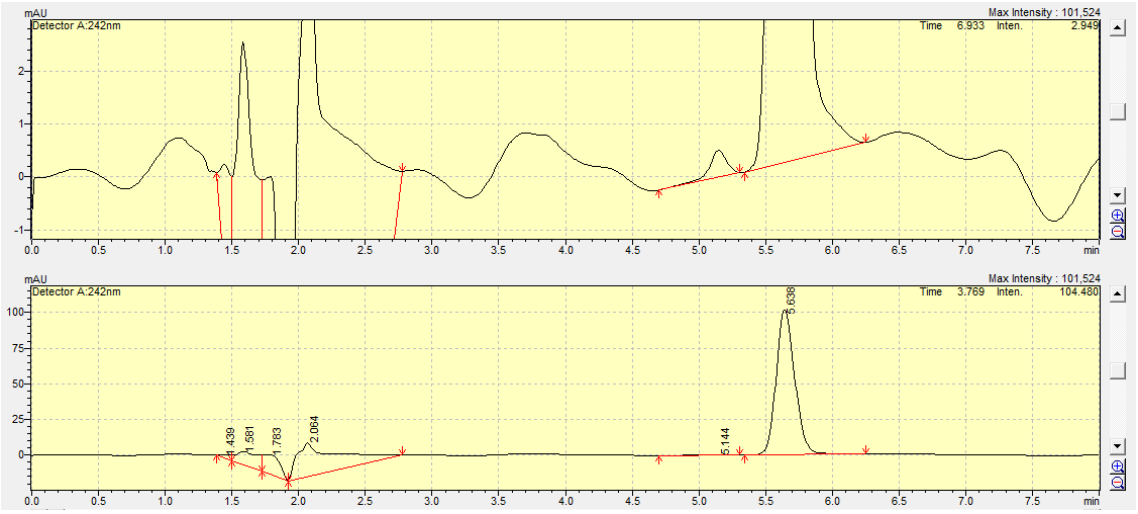

Figure 45. Chromatogram corresponding to the fifth replicate of the medicine P injection.

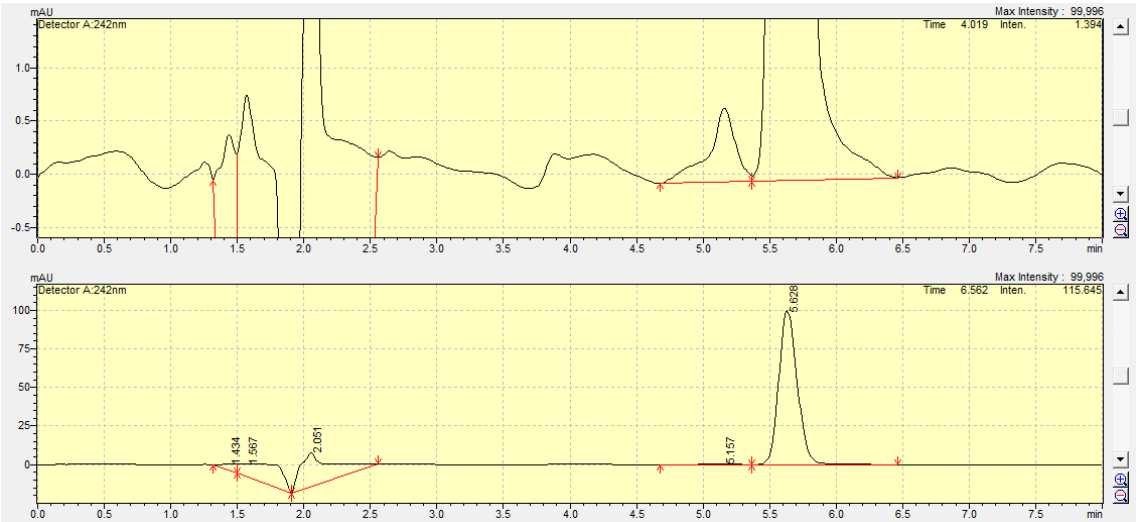

Figure 46. Chromatogram corresponding to the seventh replicate of the medicine P injection.