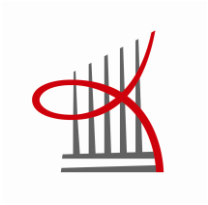

OBSERVING THE BEHAVIOUR OF AMOXICILLIN IN PHOSPHATE AND AMMONIUM BUFFERS

Juha Le Tortorec

Bachelor's Thesis
June 2014
Environmental Engineering

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ABSTRACT

Tampereen ammattikorkeakoulu
Tampere University of Applied Sciences
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LE TORTOREC, JUHA:
Observing the Behaviour of Amoxicillin in Phosphate and Ammonium Buffers

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June 2014

In modern animal husbandry veterinary medicines are used non therapeutically to promote the health and growth of animals. While economical losses due to animal sickness have been reduced this practice has also created problems. As it is generally known many pharmaceuticals have antibiotic properties and low biodegradability. Nowadays their behaviour in the environment is a common topic of discussion and research.

In this study, amoxicillin was added into phosphate and ammonium buffer solutions. The purpose was to observe how the pharmaceutical is behaving in the presence of PO_4^{3-} and NH_4^+ ions during time span of approximately 180min. 2% MeOH solutions was used as control.

Measurements were done with HPLC. Phenomenex Gemini-NX 5µm C18 110A column was used. Mobile phase was 10mM pH 6,1 KH_2PO_4 and MeCN (95:5). Wavelength was set to 227nm and flow rate to 1,5ml/min.

The chromatograms displayed two analyte peaks for each sample. Since no MS was performed, AMX could not be identified. During method development phase peak 2 displayed signs of molecular changes. However, these were not observed in the final results, suggesting that no degradation had occurred during the experiment.

Key words: veterinary medicines, amoxicillin, phosphate ion, ammonium ion, HPLC, two peaks, degradation of pharmaceutical

TIIVISTELMÄ

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Opinnäytetyö 48 sivua, joista liitteitä 3 sivua
Kesäkuu 2014

Nykyaikaisessa maataloudessa tuotantoeläimiä lääkitään ennaltaehkäisevästi tuottavuuden parantamiseksi. Vaikka eläimien sairastumisesta johtuva hävikki on vähentynyt, käytäntö voi aiheuttaa myös ongelmia. Kuten on yleisesti tiedossa, monet lääkeaineet ovat antibakteerisia ja hajoavat siksi hitaammin luonnossa. Aihe on saanut viime vuosina huomiota niin tutkimuksessa kuin mediassakin.

Tässä tutkimuksessa pyrittiin selvittämään onko fosfaatti- ja ammoniumionien pitoisuuksilla vaikutusta amoksisilliinin käyttäytymiseen liuoksessa. Työssä lääkeainetta lisättiin puskuriliuoksiin, joista sen pitoisuus määritettiin tietyin väliajoin HPLC-laitteella n. 180min ajan. Kontrollinäytteenä käytettiin 2% MeOH liuosta. Käytettävä kolonni oli Phenomenex Gemini-NX 5µm C18 110A. Eluenttina käytettiin isokraattista 10mM pH 6,1 KH_2PO_4 ja MeCN (95:5). UV-lampun aallonpituus säädettiin 227nm ja järjestelmän virtausnopeus 1,5ml/min.

Lopullisissa mittaustuloksissa esiintyi mahdollisesti kaksi eri ainetta. Näitä ei voitu tunnistaa, sillä työssä ei käytetty MS-laitetta. Menetelmää kehittäessä toinen aineista näytti hajoavan, mutta vastaavia havaintoja ei kuitenkaan tehty varsinaisissa mittauksissa. Vaikka tunnistamattomat aineet eivät pysyneet kokeen aikana täysin muuttumattomina, tulokset eivät todistettavasti viitanneet lääkeaineen suoranaiseen hajoamiseen.

Avainsanat: tuotantoeläinten lääkkeet, amoksisilliini, fosfaatti-ioni, ammoniumioni, HPLC, lääkeaineen hajoaminen

CONTENTS

ABBREVIATIONS AND TERMS

LIST OF ABBREVIATIONS AND SYMBOLS

λ	Wavelength (nm)
ADP	Amoxicillin diketopiperazine-2´, 5´ (Lamm et al., 2009)
AMX	Amoxicillin (Lamm et al., 2009)
A_{peak}	Peak area
A_{re}	Relative area
$C_2H_7NO_2$	Ammonium acetate
CO_2	Carbon dioxide
H^+	Hydrogen ion
H_2O	Water
H_3O^+	Hydronium ion
HPLC	High performance liquid chromatography
k	Slope
KH_2PO_4	Potassium dihydrogen phosphate
LC	Liquid chromatography
LC/MS	Liquid chromatography mass spectrometry
M	Molarity (mol/l)
MeOH	Methanol
MeCN	Acetonitrile
MS	Mass spectrometry
NaH_2PO_4	Sodium dihydrogen phosphate
Na_3PO_4	Trisodium phosphate
NaOH	Sodium hydroxide
NH_3	Ammonia
NH_4^+	Ammonium ion
OH^-	Hydroxide ion
OTC	Oxytetracycline (Chemical Book, 2010)
p	Pressure (bar)
p_{max}	Maximum pressure (bar)
PO_4^{3-}	Phosphate ion
ppm	Parts per million (mg/l)

R^2	Correlation
RT	Retention time (min)
SMZ	Sulphamethoxazole (Chemspider, 2014, Sulphamethoxazole)
SPE	Solid phase extraction
T	Temperature (°C)
t	time (min)
TYL	Tylosin
UHP	Ultra high purified
UV	Ultraviolet
V	Volume (ml)
$V_{injection}$	Injection volume (µl)

1 INTRODUCTION

Usage of veterinary medicines in modern agriculture is common thorough the whole world. In animal husbandry pharmaceuticals and hormones are non therapeutically given to livestock in order to improve their health and growth (Animal Health Institute, 2014; Martin et al., 2010; YLE 1, 2013; The Wall Street Journal, 2013).

A study published in *Journal of Antimicrobial Chemotherapy* shows that in year 2007 18-188mg of veterinary antibacterial agents were sold per a kilogram of meat (swine, cattle and poultry) produced within 10 selected European countries (Grave et al., 2010). Netherlands and France had the highest amount, while Norway, Sweden and Finland had the lowest. Out of the EU countries Sweden had the lowest value (~25mg/kg).

While the modern practice has prevented disease and thus minimised losses (YLE 1, 2013), specialist of different fields criticise the carefree attitude of using livestock medication for non therapeutic purposes (prevention, growth improvement). Elstrøm from the Norwegian Public Health Institute stated that ”there is a direct link between the use of antibiotics in livestock and resistant bacteria in humans” (EurActiv, 2014). Continuing with the current practices could potentially lead to formation of antibiotic resistant super bacteria (Mackie, 2011; RT, 2014).

The non metabolised pharmaceuticals are excreted by the animals and can be found in trace amounts from the faeces and urine. Eventually these will end up into the soil and natural water bodies.

There is reason to believe that these compounds have certain persistence in the environment. Biological degradation may occur slowly due to the natural antibiotic properties of certain compounds. Furthermore, as the molecules are complex, unknown chemical reactions between different compounds may occur. This way new, potentially more hazardous substances can be formed (YLE 2, 2013).

While the final fate of these substances in the environment remains unclear (YLE 2, 2013), it is heavily researched on nowadays. During recent years the issue has been getting more attention in the media, raising public awareness (YLE 3, 2013).

2 BACKGROUND

The basis for this thesis work is laid on two scientific papers. These were *Factors affecting the degradation of amoxicillin in composting toilet* by Kakimoto and Funamizu (2006), and *Degradation of veterinary medicines in composting process of livestock manure* by Ishiduka (2013).

Kakimoto and Funamizu had discovered that the degradation of amoxicillin had a relation to the concentration of phosphate, ammonium and hydroxyl ions in composting environment. It was concluded that these were factors controlling the degradation process.

In her work Ishiduka noticed that degradation of the selected veterinary medicines (including amoxicillin) occurred regardless of biological activity. She concluded that the cause of degradation was chemical reactions.

During my internship period in Japan in spring 2013, I applied Kakimoto's and Funamizu's method (although modified) on three of Ishiduka's pharmaceuticals (SMZ, OTC and TYL). The aim of my work was to find out if phosphate and ammonium ions had an effect on the degradation rate.

While in Japan, I discussed with Kakimoto, Funamizu and Ishiduka in person. In our conversation I gained a lot of practical information about the setup of the experiments itself, knowledge that cannot be found from books or Internet. This was very beneficial regarding my work. After several trials, I was able to conduct the experiment. Sample preparation was done by using a SPE method. Analysis was carried out by using LC/MS.

The results failed to show proper degradation lines for the pharmaceuticals as the level of detected pharmaceuticals was varying unnaturally. While there had been some issues with the LC/MS in the past, this was not the probable cause. Mistakes in the SPE process were suspected.

Regardless of this, I presented my findings in a seminar at the end of my internship period. After my presentation I received several comments. A Ph.D. candidate in my laboratory pointed out that it would have been wiser to first see whether I could repeat Kakimoto's and Funamizu's experiment with amoxicillin before applying the method for any other pharmaceuticals. Realized that this was elementary, I concluded that any further work should focus on that.

After discussing the continuation of the research with my teachers, I got my Bachelor's thesis topic accepted. In January 2014 I started working in the laboratories at Tampere University of Applied Sciences.

3 THEORY

3.1 Amoxicillin

Amoxicillin ($C_{16}H_{19}N_3O_5S$; Figure 1) is a semi synthetic antibiotic substance belonging to the penicillin group (Chemspider, 2014, Amoxicillin; Crosta, 2012). It is used in the treatment of several bacterial infections, including "infections of the ears, nose, throat, urinary tract, and skin" (MedlinePlus, 2010). More serious microbial infections, such as the ones caused by E. coli and salmonella can also be treated with amoxicillin (Drugs, 2014).

While the form, regulations, practice and dosage varies, amoxicillin is therapeutically used to treat both human and animal disease.

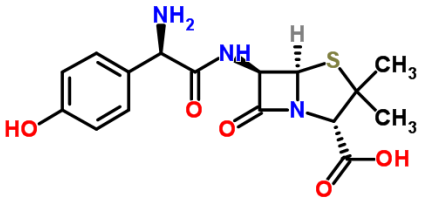

Figure 1. Amoxicillin molecule (ChemSpider, 2014, Amoxicillin).

3.2 Buffers

The pH of any solution is determined by the ratio between H_3O^+ and OH^- ions (Lewis et al., 2001; pp. 286-287). This ratio can be easily changed by adding acid or base, thus moving the equilibrium.

Solutions with the ability to resist this change are called buffers. They contain “a weak base and one of its salts, or a weak acid and one its salts” (Lewis et al., 2001; p. 298). These solutions have components capable of binding H^+ ions and changing OH^- ions into water molecules (Clark, 2002, buffer solutions). Naturally, this ability is limited, and is determined by the capacity of the buffer (Lewis et al., 2001; p. 300).

Buffers are naturally present in soil and water bodies, but also in living cells and body liquids such as blood, where changes in pH would be catastrophic (terracult; Lewis et al., 2001, p. 297).

3.3 Chromatography

3.3.1 Principle

Chromatography is a method for separating different components from mixtures (Clark, 2007, thin layer chromatography). While there are several types of chromatographic applications in use, the operating principle is always the same (Clark, 2007, thin layer chromatography).

Basically a chromatograph requires two things to function: a mobile phase and a stationary phase. The purpose of the mobile phase is to transfer the sample through the stationary phase. The function of the stationary phase is to separate the analytes (target compounds) from each other utilizing the differences in their chemical properties (Clark, 2007, thin layer chromatography).

3.3.2 HPLC

High performance liquid chromatography is a sophisticated application of liquid chromatography. It utilises high pressure (up to 400bar) making the separation process much faster than in simple chromatographic applications using only gravitational force

(Clark, 2007, high performance liquid chromatography - HPLC). Figure 2 shows a simplified design of a HPLC unit.

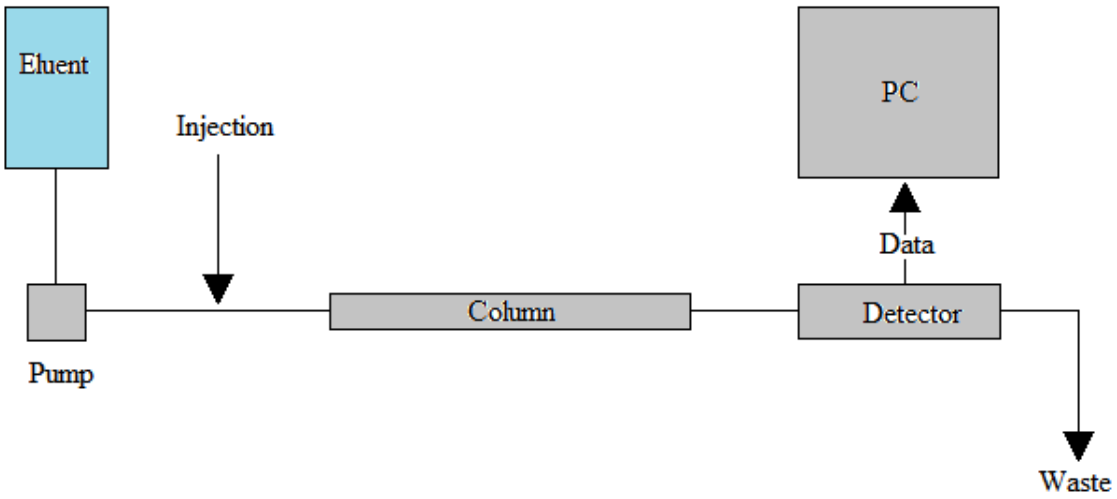

Figure2. An illustration of HPLC unit.

Eluent is the liquid functioning as the mobile phase in LC. Depending on the method, it can be either isocratic or gradient. The composition of an isocratic eluent remains constant during the run, while gradient changes steadily (Types of HPLC). This can be beneficial, if there is more than one target compound is to be measured.

The column used in HPLC is a cylindrical metallic tube with silica surface on the inside (Clark, 2007, thin layer chromatography). This functions as the stationary phase in LC, separating the analytes from the eluent. The separation process is very delicate and requires careful control of conditions (explained further on).

Separation process

Based on the nature of the stationary phase and separation process, the majority of all liquid column chromatography applications can be classified into two categories: size exclusion chromatography and ion exchange chromatography (Types of HPLC). The

latter one is further divided into normal phase and reversed phase chromatography (Types of HPLC).

Size exclusion chromatography

"In size exclusion chromatography the column is filled with material having precisely controlled pore sizes" (Types of HPLC). The pores function as filters, controlling the size of molecules that can travel through them. Larger molecules are rapidly washed through the column, while the smaller travel through the pores, thus taking longer time to reach the end of the column (Types of HPLC).

Ion exchange chromatography

The separation process in ion exchange chromatography is based on the opposite charges between the stationary phase and sample ions (Types of HPLC). "The stronger the charge on the sample, the stronger it will be attracted to the ionic surface and thus, the longer it will take to elute" (Types of HPLC).

In normal phase chromatography the stationary phase is polar (e.g. silica) and the mobile phase non polar. This retains the polar compounds inside the stationary phase, while the non polar ones will stay in the mobile phase and travel through faster (Clark, 2007, high performance liquid chromatography - HPLC).

Reversed phase chromatography is the exact opposite of normal phase. The stationary phase is non polar (silica modified with long hydrocarbon chains) and the mobile phase polar (Clark, 2007, high performance liquid chromatography – HPLC). Reversed phase chromatography uses either C8 or C18-columns as stationary phase (Clark, 2007, high performance liquid chromatography – HPLC).

Retention time

The time it takes for a compound to pass through the column and reach the detector is referred to as retention time. RT for a particular compound will depend on column temperature, flow rate (affects directly the pressure), how well it is dissolving in the eluent and how much it is reacting with the surface of the column (Clark, 2007, thin layer chromatography). If these conditions stay exactly the same, theoretically speaking no variations in RT for a particular compound should occur.

Detector

After the separation process, the analytes reach the UV detector (Figure 2). UV wavelength radiation (40-400nm) is emitted from the lamp and directed to travel through the eluent carrying the analytes (Deman, 2011; Figure 3). The amount of radiation absorbed will be measured, and the data is sent to the computer.

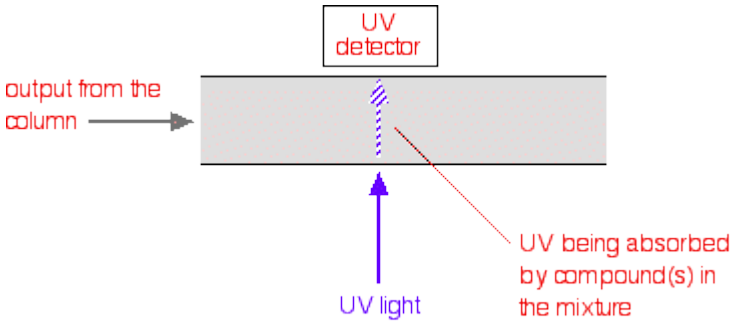

Figure 3. A picture illustrating how the UV radiation is directed towards the detector (Clark, 2007, high performance liquid chromatography - HPLC).

Integration of chromatogram

The output of the detector is referred to as chromatogram (Figure 4) (Chromatography - The Chromatogram), which is basically the baseline with peaks for the detected compounds. Based on the setting of the computer, these peaks are integrated and yield an area value.

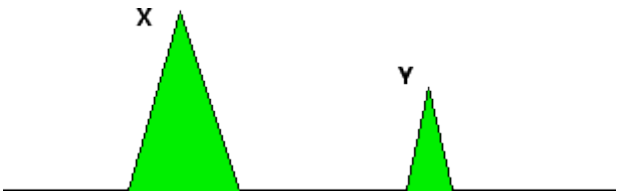

Figure 4. Simplified chromatogram peaks (Clark, 2007, high performance liquid chromatography - HPLC).

“According to the Beer-Lambert Law, absorbance is proportional to concentration -- as long as the solutions are dilute“ (Clark, 2007, using UV-visible absorption spectra). In other words, higher the area of the peak means higher concentration of analytes (Clark, 2007, high performance liquid chromatography - HPLC).

4 OBJECTIVE

The objective of this study was to find out whether PO_4^{3-} and NH_4^+ ions have an effect on the degradation of amoxicillin at constant pH conditions. This was done by performing a similar experiment to Kakimoto's and Funamizu's one.

There were four stages in the work:

The first stage was to plan and develop an experiment where PO_4^{3-} and NH_4^+ ions are present in a solution at constant pH conditions. After this it was necessary to find a method capable of measuring the pharmaceutical to be studied from these solutions. This was applied to the selected pharmaceutical (AMX). Based on the results conclusions were made.

5 METHOD

The principle of the new method was to spike the pharmaceutical in a solution and make consecutive injections in HPLC.

5.1 Stock solution

Stock solution for amoxicillin was prepares by weighting 11.5 mg of amoxicillin trihydrate (~10mg of pure amoxicillin) in a weighting vessel. The pharmaceutical was washed from the weighting vessel into a 10ml volumetric flask with MeOH. 500µl of UHP water was added by using an automatic pipette. After this the flask was filled up to the line with MeOH. The flask was then sealed and shaken until amoxicillin was completely dissolved.

The final solution had 1000ppm of pure amoxicillin dissolved in 95% MeOH. The solution was poured into a 10ml brown glass laboratory bottle. The bottle was sealed, covered with aluminium foil and stored in freezer (-17°C).

Methanol was used as the solvent because of two reasons. One: amoxicillin is reported to be soluble in methanol (see text further on). Two: MeOH solution can be stored in a freezer in liquid form and is easy to spike.

As it is commonly known, absolute methanol is very hydrophilic and evaporates easily. In order to reduce changes in volume during storage, MeOH used was diluted from 100% to 95%. Storing the bottle in freezer further reduced this effect. While the freezing point of the mixture is higher than that of absolute methanol, this did not matter since very low temperature was not used (-17°C).

When preparing the sample it was noticed that the trihydrate form was dissolving poorly in 95% MeOH. The reason for this remained unclear, since amoxicillin is known to be dissolving in water and methanol (Merck Index, 2001, p. 96). Other antiobiotics, such as SMZ, OTC and TYL dissolve in MeOH very quickly.

As the prepared stock solution was very concentrated (1000ppm), it was possible to use a low spiking volume (200µl per 10ml) and still have a relatively high concentration of pharmaceuticals in the sample (20ppm). Higher concentration in the sample made detection easier.

5.2 Diluents

The diluents were prepared in the described manner.

5.2.1 Methanol solution

The 2% MeOH solution was prepared by filling a 10ml volumetric flask with UHP water almost until the line. 200µl of MeOH was added by using an automatic pipette. After this the flask was filled until the line with UHP water, sealed and shaken.

5.2.2 Phosphate buffer

50ml of 200mM Na_3PO_4 solution and 50ml 200mM NaH_2PO_4 solution were prepared. NaH_2PO_4 solution was emptied into a beaker. While constantly stirring and measuring the pH, Na_3PO_4 solution was added with pasteur pipette until pH 8,5 was reached.

5.2.3 Ammonium buffer

50ml of 200mM $C_2H_7NO_2$ solution and 50ml 200mM NH_3 solution were prepared. $C_2H_7NO_2$ solution was emptied into a beaker. While constantly stirring and measuring the pH, NH_3 solution was added with pasteur pipette until pH 8,5 was reached.

The ammonia calculation was made by using a table found from Internet (Chemistry Archive). Due to volatilisation of ammonia in basic conditions (Richard, 1996), the readymade buffer solution was stored in fridge.

5.3 UV spectrum

UV spectrum was determined for AMX by using UV/VIS spectrometer (Figure 5). The values were measured from 1% MeOH solutions with pharmaceutical concentration of 10ppm. Total of three measurements were done. 1% MeOH solution was used as control.

Figure 5. PerkinElmer instruments UV/VIS Spectrometer unit.

5.4 Sample preparation

Each sample solution containing pharmaceutical was prepared three minutes before the injection in order to give accurate result for the initial (0min) measurement.

A 10ml volumetric flask was filled almost until the line with diluent. 200µl of pharmaceutical stock solution was spiked by using an automatic pipette. The flask was filled until the line with diluent, sealed and shaken for one minute.

After this, the solution was poured into a small beaker. Some of the solution was transferred into a syringe (1-2ml). Air was removed by turning the syringe vertically up and tapping the side lightly so that the air bubble moved to the surface. Some solution was pressed out from the solution, at the same time removing the air bubble.

Next a syringe filter was placed to the end of the syringe. The solution was lightly pressed through the filter into a 2ml screw cap via. The vial was sealed and placed on the autosampler rack, ready to be automatically injected.

5.5 HPLC method

For this work, an existing HPLC method for amoxicillin was modified (Lunn, 2000, p. 385) (see method settings). Analysis was carried out by using Agilent HPLC 1100 Series unit (Figure 7). The separation process was reversed phase chromatography. A C18 column was used with guard column attached (Figure 6).

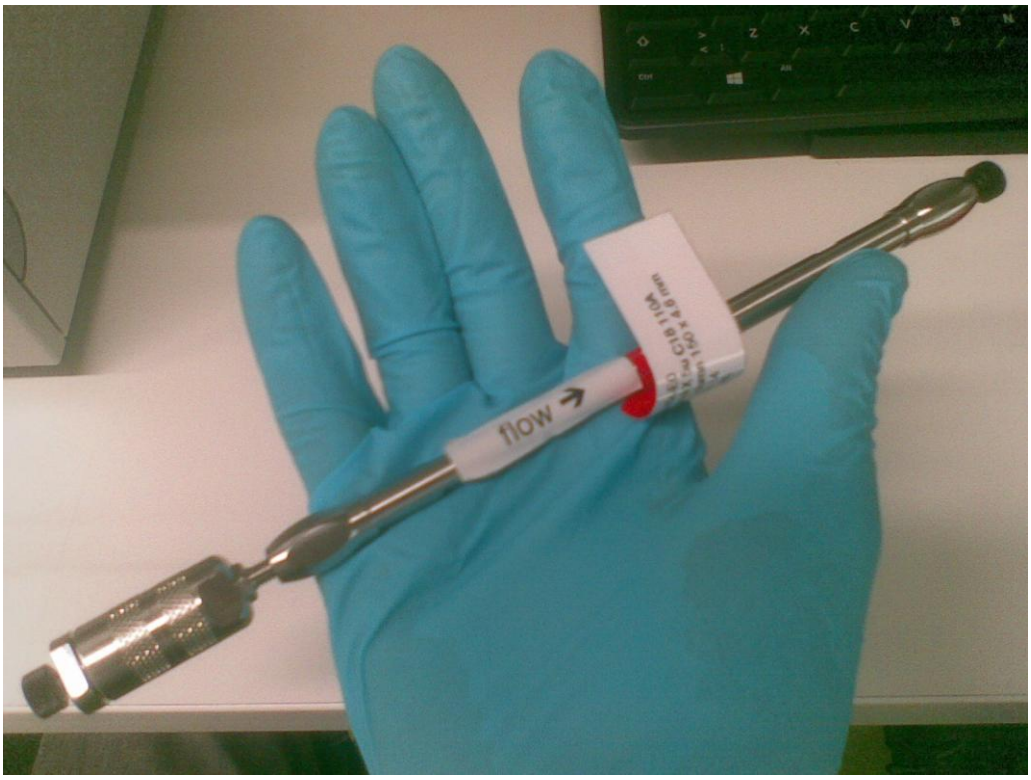

Figure 6. Phenomenex Gemini-NX 5µm C18 110A column with a guard column attached.

Figure 7. Agilent HPLC 1100 Series unit.

5.5.1 Eluent

Eluent A was made by preparing 10mM solution of KH_2PO_4 solution and setting the pH to 6,1 with NaOH (Table 1). The solution was filtered by using a vacuum filtration unit in order to reach HPLC grade (Figure 8). MeCN was used as eluent B.

Table 1. Mobile phase used in amoxicillin measurements (isocratic).

	A	B
Eluent	10mM KH_2PO_4 pH 6.1 (NaOH)	MeCN
v/v (%)	95	5

Figure 8. Vacuum filtration unit used for preparing eluent A.

5.5.2 Experimental conditions

HPLC method settings can be seen from table 2.

Table 2. Method settings for HPLC. *) Changed from the original method (Lunn, 2000, V2, p. 385).

Column	Phenomenex Gemini-NX 5µm C18 110A *
V (injection)	20µl
Flow rate	1.5ml/min *
T (column)	Ambient (~20°C)
t	15min or 17min
p (max)	250bar

5.5.3 Integration settings

The integration settings of the HPLC unit are presented in table 3. The limits were set quite low on purpose in order to get all peaks integrated.

Table 3. Integration settings for chromatogram analysis.

Slope sensitivity	5
Min. peak width	0.05min
Min. peak height	0.1mAU
Min. peak area	1

5.6 Reporting and data processing

The used HPLC method reported the results of the measurements in PDF and TXT format. Chromatograms were screen captured from the PDF files. Raw data from TXT files was used to produce the figures. MO Excel was used for data processing.

6 RESULTS

The results of the experiments are presented in this chapter.

6.1 UV spectrum

The UV spectrum for AMX can be seen from figure 9.

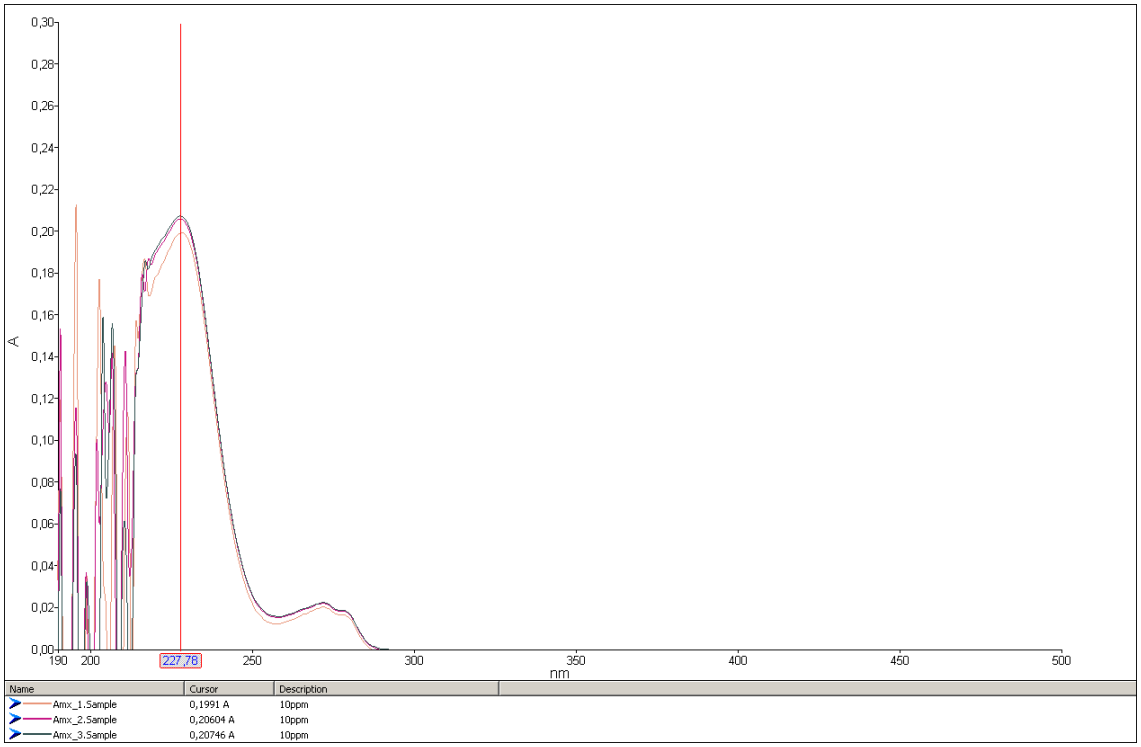

Figure 9. UV spectrum of amoxicillin.

According to the measurements amoxicillin reaches maximum absorbance at approximately 228nm. Wavelength of 227nm was used in the HPLC measurements. This is very close to the previously reported values (de Abreu et al., 2003; Drug Future, 2013; Jang Lee et al., 2013).

6.2 HPLC

The results for the HPLC measurements are presented here. Due to limitations in using of analytical equipment, some measurements were done with fewer injections than others. In order to compare the figures better, linear lines estimating the probable development of the scatter plots were drawn by using k value.

6.2.1 Chromatograms

Every time a new measurement begun the produced chromatogram received some disturbance. This originated from the injection itself, as well as UV absorbance of solution the analytes were dissolved in (diluent). This part of the chromatogram was referred to as noise, and was excluded from the data analysis.

Figures 10, 13 and 16 are examples of chromatograms from injections containing only the sample diluent without any pharmaceuticals. This information made it possible to estimate the effect the diluents used had on the AMX measurements, thus improving the accuracy of the method.

The sample injection was compared against the chromatogram produced by the diluent. While these could not be overlapped by the program, it still made it possible to identify the peaks originating from the spiked pharmaceutical. This is true, since (as far as it is known) only one new substance has been introduced.

The chromatogram in figure 10 is produced by 2% MeOH diluent injection. While the baseline is not fully stabilised, the peak produced by diluent is visible.

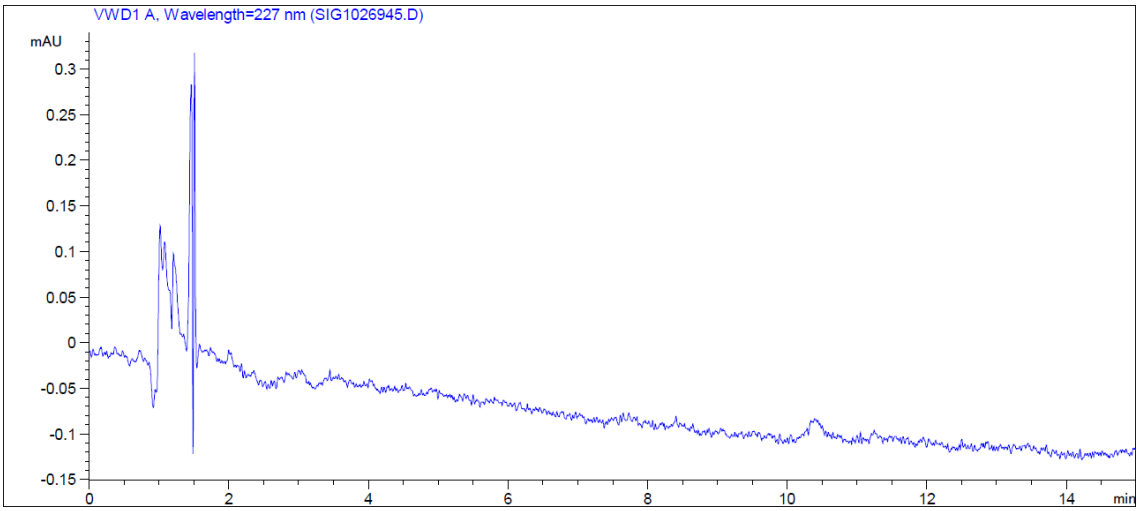

Figure 10. 2% MeOH injection (SIG1026945).

Figure 11 demonstrates the chromatogram in produced by 2% MeOH+20ppm AMX injection at 0min. Peak 1 is sharp and high, staying nearly constant during the measurements. Peak 2 on the other hand, is short and wide. Towards the end, the peak is splitting (Figure 12).

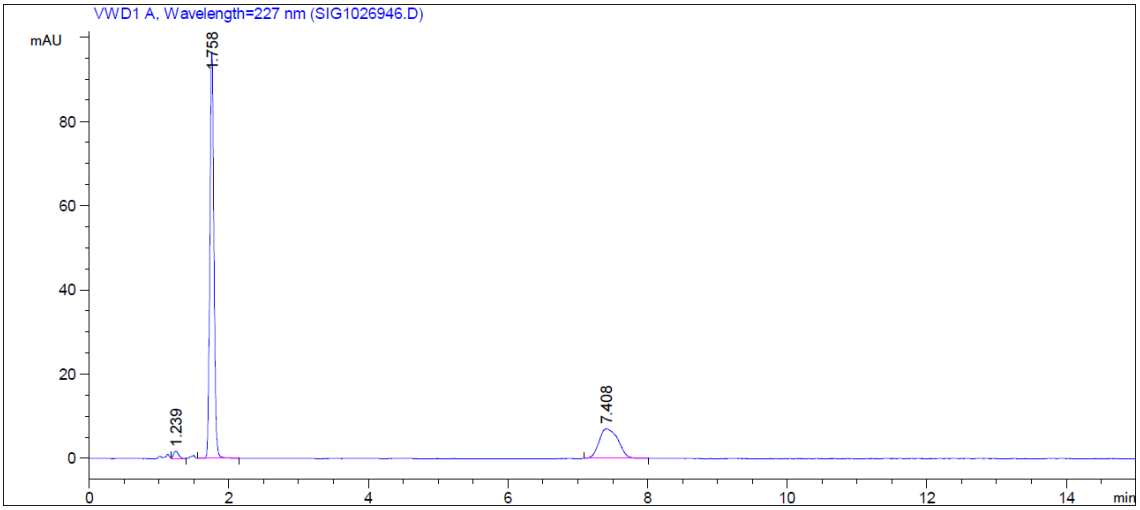

Figure 11. 2% MeOH+20ppm AMX injection (0min) (SIG1026946).

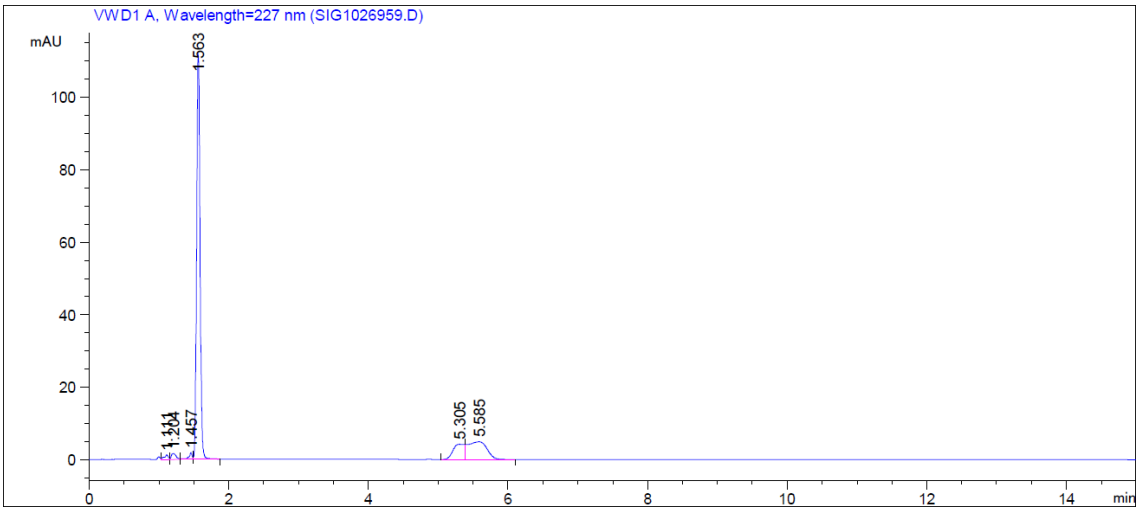

Figure 12. 2% MeOH+20ppm AMX injection (195min) (SIG1026959).

The chromatogram in figure 13 represents 200mM pH 8,5 phosphate buffer+2% MeOH injection. Baseline is good and diluent peaks clearly visible.

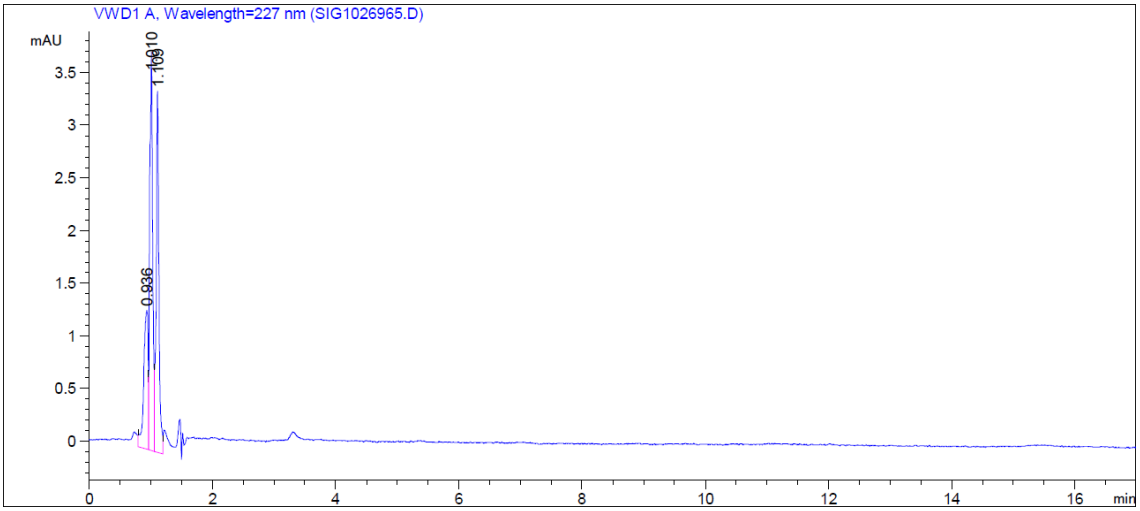

Figure 13. 200mM pH 8,5 phosphate buffer+2% MeOH injection (SIG1026965).

Figure 14 demonstrates the chromatogram produced by 200mM pH 8,5 phosphate buffer+2% MeOH+20ppm AMX injection at 0min. Again, peak 1 is very sharp, high and stays constant. Peak 2 is fronting in the beginning, but seems to turn vertical and sharpen later on (Figure 15).

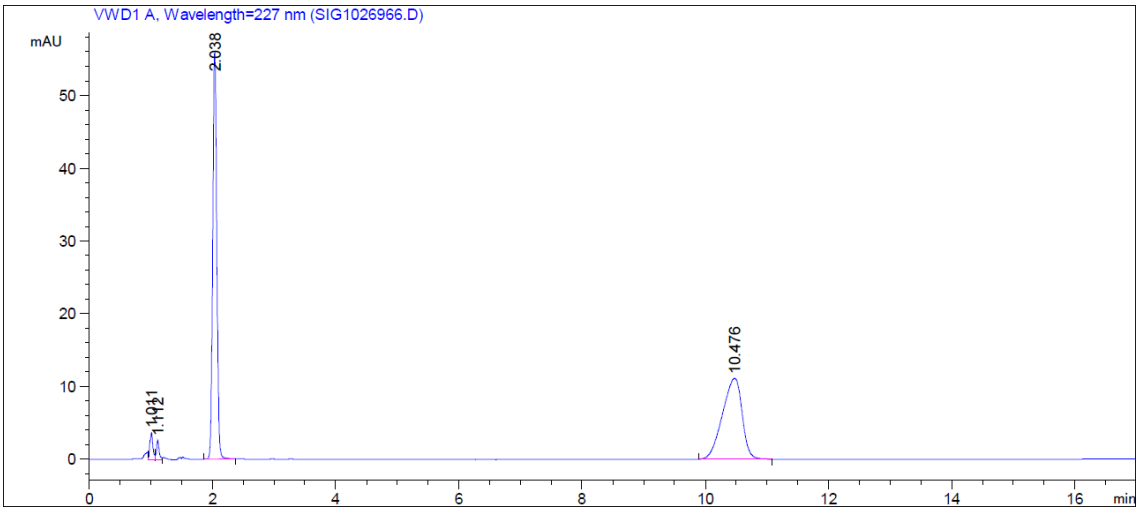

Figure 14. 200mM pH 8,5 phosphate buffer+2% MeOH+20ppm AMX injection (0min) (SIG1026966).

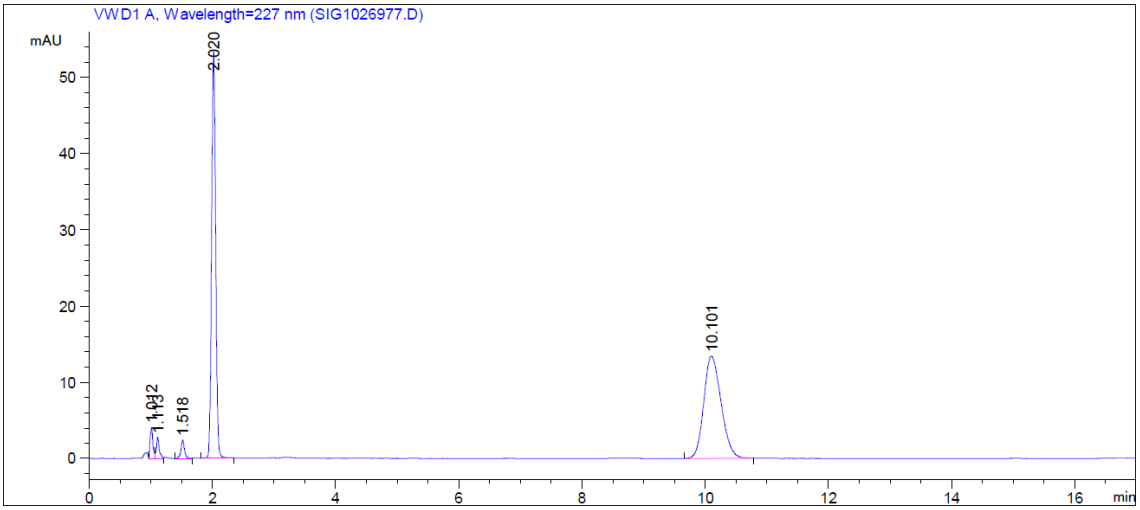

Figure 15. 200mM pH 8,5 phosphate buffer+2% MeOH+20ppm AMX injection (187min) (SIG1026977).

Next is the chromatogram for 200mM pH 8,5 ammonium buffer+2% MeOH injection (Figure 16). Baseline is very good and diluent peaks clearly visible.

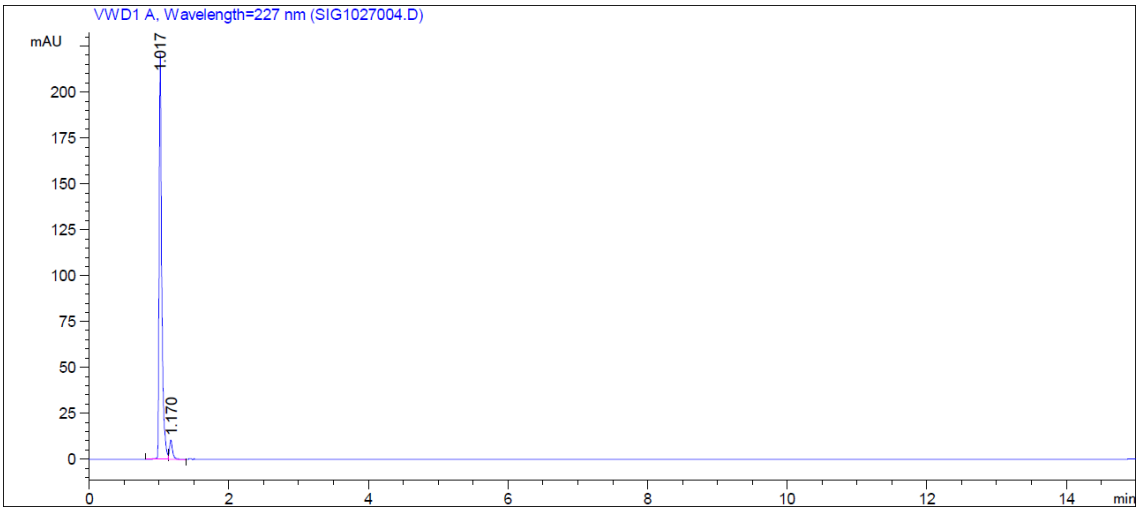

Figure 16. 200mM pH 8,5 ammonium buffer+2% MeOH injection (SIG1027004).

Figure 17 demonstrates the chromatogram produced by 200mM pH 8,5 ammonium buffer+2% MeOH+20ppm AMX injection at 0min. Surprisingly, peak 1 is clearly shorter than in other treatments. The peak stays sharp and nearly constant. Peak 2 resembles an equilateral triangle, and seems to be slightly broadening to the right hand side (Figure 18).

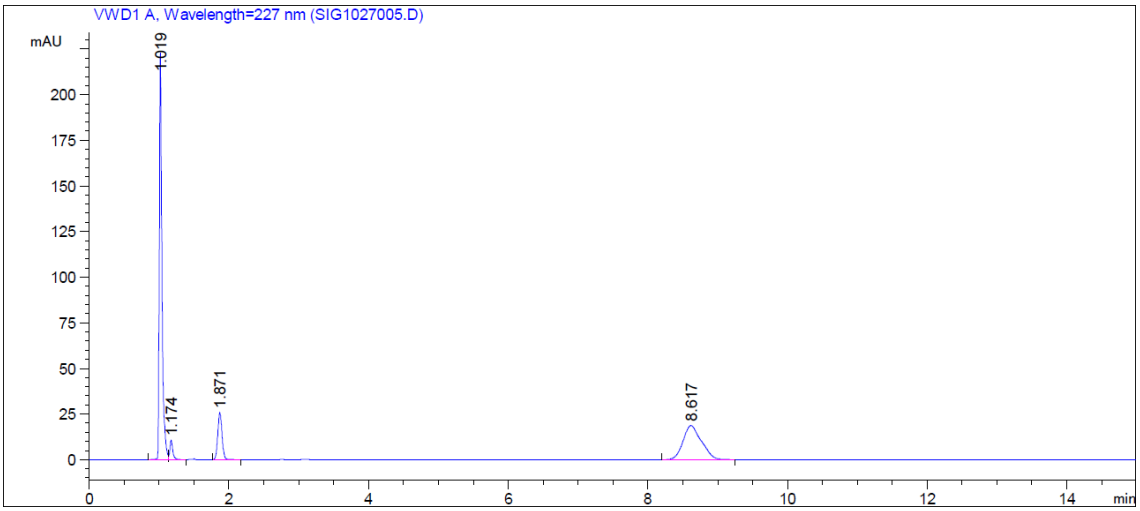

Figure 17. 200mM pH 8,5 ammonium buffer+2% MeOH+20ppm AMX injection (0min) (SIG1027005).

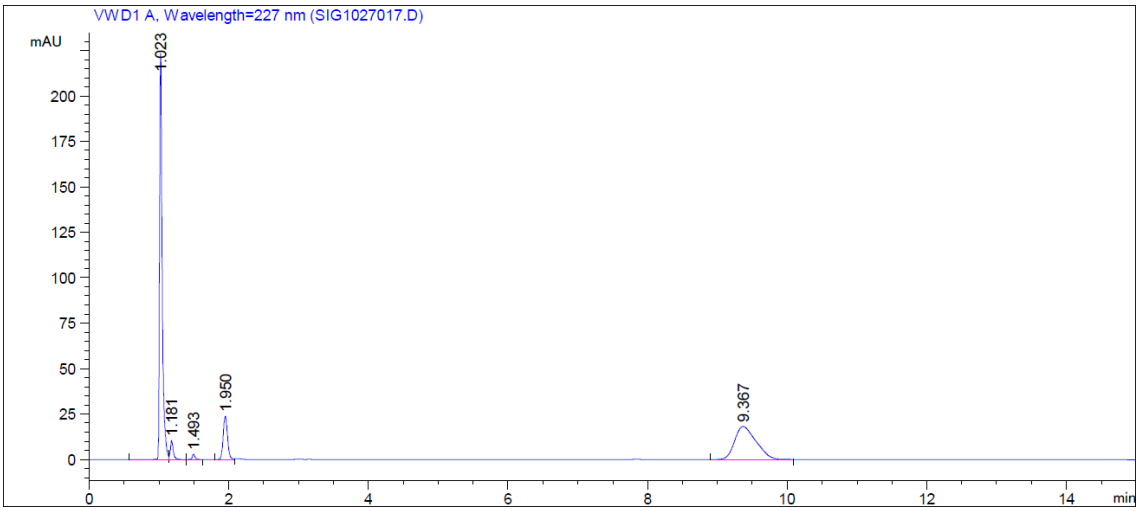

Figure 18. 200mM pH 8,5 ammonium buffer+2% MeOH+20ppm AMX injection (180min) (SIG1027017).

As can be seen from figures 10, 13 and 16, the diluent chromatograms have almost no peaks at RT>1,5min. Same peaks can be found from sample chromatograms.

Figures 11, 12, 14, 15, 17 and 18 represent the sample injections. As can be seen from the chromatograms, aside from the diluent, two peaks were detected. However, the way these are divided and situated (RT, area and shape) is different in each. The peaks were labelled simply as peak 1 and peak 2, based on lowest RT. Regardless of their naming, peak x (1 or 2) is not necessarily the same compound in each treatment. The name simply indicates the order in which they were detected.

6.2.2 Retention time

The following scatter plots (Figures 19, 20 and 21) represent RT of the peaks detected from consecutive injections of diluent+AMX.

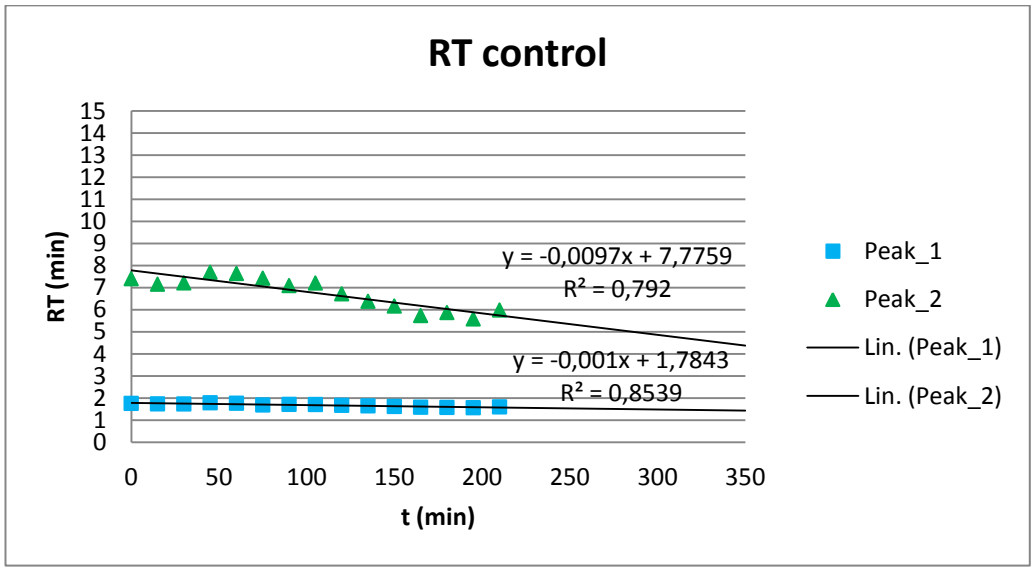

Figure 19. RT of peak 1&2 in 2% MeOH solution.

Figure 19 shows that in control treatment RT_{peak1} is ~1,8min and remains practically constant (k=-0,001). While RT_{peak2} is ~7,4, it does not stay constant.

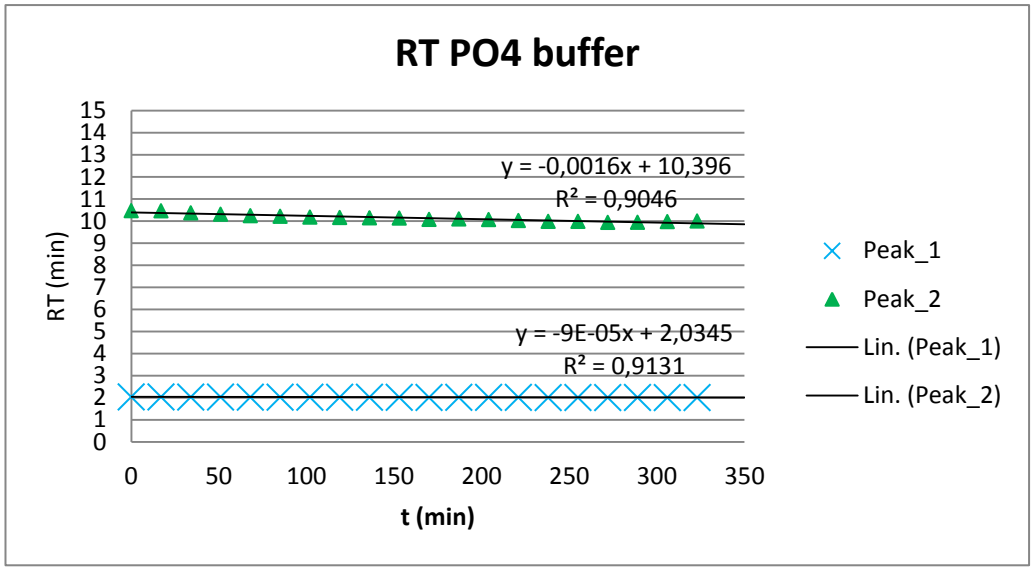

Figure 20. RT of peak 1&2 in 200mM pH 8,5 phosphate buffer + 2%MeOH solution.

As figure 20 represents, in the phosphate buffer treatment both peak 1 and peak 2 have stable RT (k_{peak1}=-9E-05, k_{peak2}=-0,0016). RT_{peak1} is 2min and RT_{peak2} ~10min.

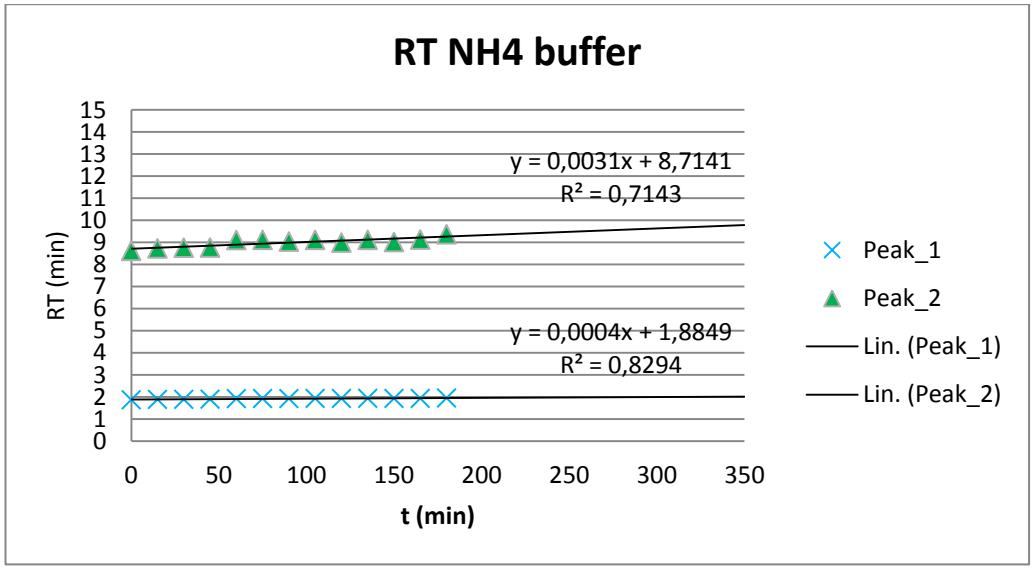

Figure 21. RT of peak 1&2 in 200mM pH 8,5 ammonium buffer+2% MeOH solution.

Figure 21 displays that in ammonium buffer treatment RT_{peak1} remains constant at ~2min (k =0,0004). While RT_{peak2} is ~8,6min, it does not stay stable.

6.2.3 Peak area

The following scatter plots (Figures 22, 23 and 24) represent the area (proportional to amount, see theory) of the peaks detected from consecutive injections of diluent+AMX.

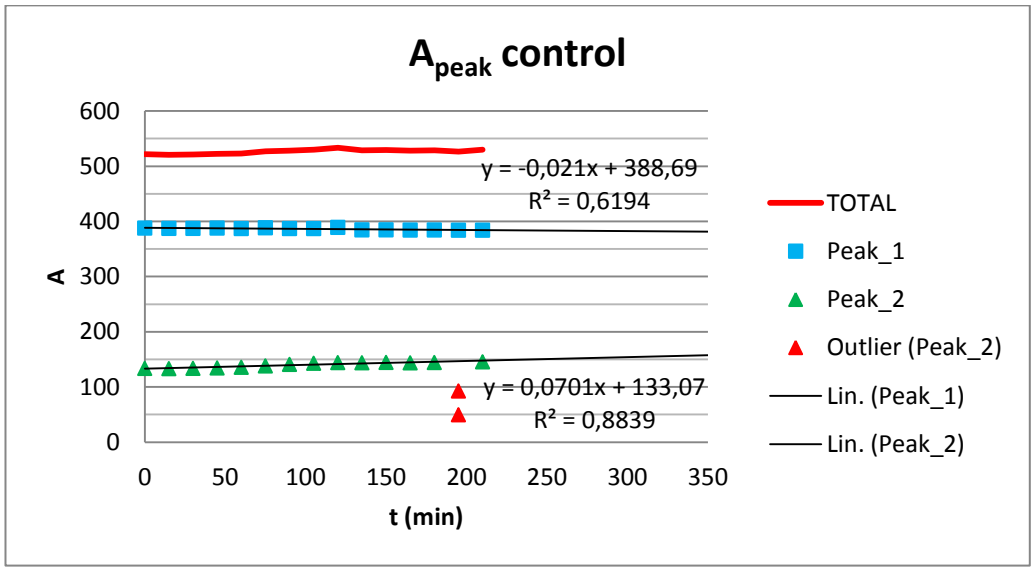

Figure 22. Area of peak 1&2 in 2% MeOH solution.

As can be seen from figure 22, the peaks were clearly divided in terms of area. Peak 1 has an area of ~400 at 0min, and seems to be slightly decreasing (k =-0,021). Area of peak 2 starts at ~125 and is increasing (k=0,0701). Towards the end, the peak starts splitting and got integrated as two separate ones (Figure 12). Since this had not occurred in the other chromatograms, the measurement was excluded. A_{total} (A_{peak1} + A_{peak2}) stays nearly constant.

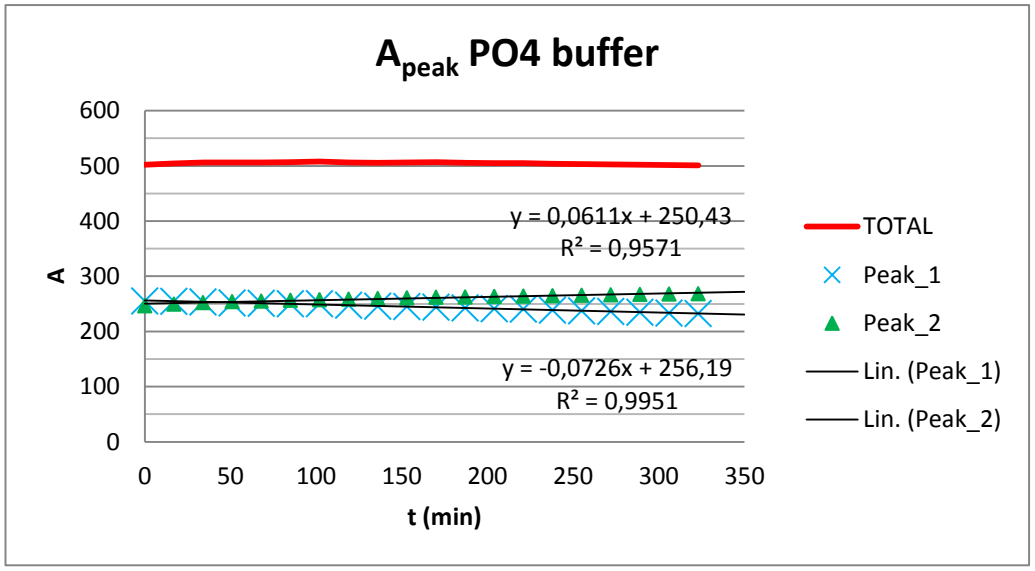

Figure 23. Area of peak 1&2 in 200mM pH 8,5 phosphate buffer+ 2% MeOH solution.

In figure 23, A_{peak1} and A_{peak2} are practically the same at 0min. A_{peak1} is decreasing (k=-0.0726) while A_{peak2} is increasing (k =0.0611). A_{total} stays constant.

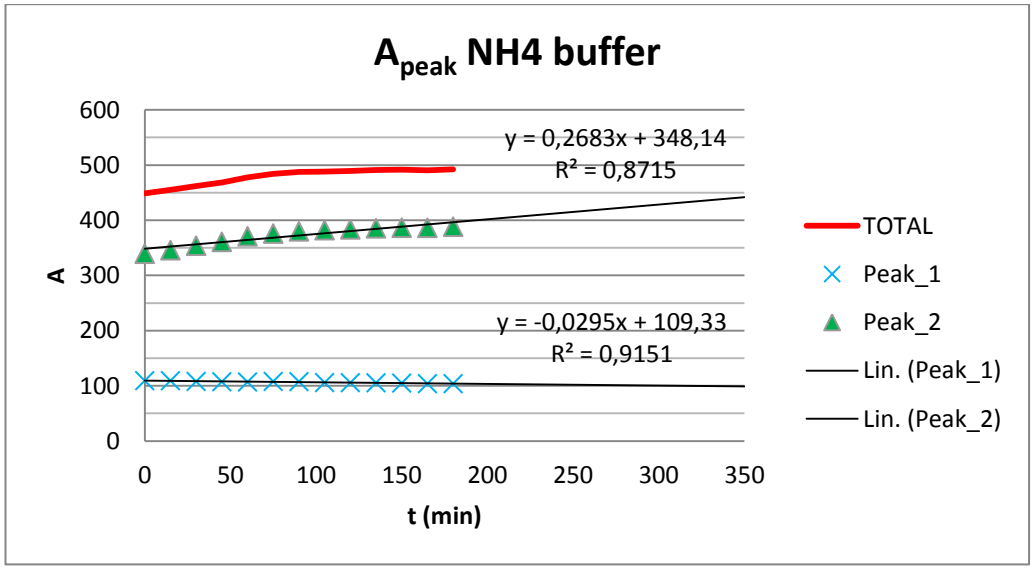

Figure 24. Area of peak 1&2 in 200mM pH 8,5 ammonium buffer+2% MeOH solution.

In figure 24, A_{peak1} is ~100 at 0min and seems to be slightly decreasing (k =-0,0295). A_{peak2}~340 at 0min and is rapidly increasing (k=0,2683). A_{total} is increasing, but gets stabilised at ~500.

7 DISCUSSION

7.1 Separate peaks

As the chromatograms display, every sample injection produced two peaks. This suggests one of two things.

Either there are two compounds present in each sample. While one of these might have been AMX, the other one remains unknown. Since no MS was performed, it was not possible to verify that AMX was detected in its unchanged form, let alone to identify the other compound. In this case, the phenomenon could be explained by impurities, contamination or degradation.

The other possibility is that for some reason AMX appears as two peaks in the chromatograms. This could be due to partial ionisation of the analyte.

7.1.1 Impurities

It is possible that one of the peaks represents AMX and the other impurities originating from the manufactured trihydrate form. In *Amoxicillin Summary Validation Report* (2013) 11 impurities were detected and identified from amoxicillin trihydrate. A_{re} was varying per impurity (>12%) but it was surprisingly large when compared to AMX (A_{re} ~46%).

As the figures 22, 23 and 24 show, A_{re} of peak 1 and peak 2 varied between different treatments. In 200mM pH 8,5 phosphate buffer+2%MeOH+AMX solution peak 1 and peak 2 have practically equal area at 0min (Figure 23). This suggests that impurities are not the cause for two peaks to appear.

One way to reduce the amount of impurities would be to use SPE in the sample preparation. While this increases the workload of the method it should reduce the noise

in the chromatogram and so improve accuracy. However, the usage of automated SPE system is recommended, since the manual method carries higher risk of human error.

7.1.2 Contamination

Considering the simplicity of sample preparation procedure and the used working practices, contamination seems a very unlikely cause for separate peaks to appear in the chromatogram.

7.1.3 Degradation products

On the other hand, there is a possibility that the separate peaks originate from degradation products of AMX. One pathway of amoxicillin degradation in an aqueous medium is transformation into AMX-penicilloic acid, and then into ADP (Lamm et al., 2009). ADP is further divided into two isomers (Lamm et al., 2009). Both AMX and ADP have similar UV spectrum, meaning that if these were present in the same sample, both would detected (Lamm et al., 2009). However, in order to verify this MS is necessary.

7.1.4 Partial ionisation

Another explanation is that the peaks represent the same compound in ionised and non ionised form. As it is commonly known, ionisation of analyte in the mobile phase can lead to peak splitting (Basniwal, Jain, 2013). When the ionisation time is considerably longer than the retention time for the compound, it is possible that two separate peaks will appear (Dolan, 2013). In reverse phase chromatography, the first peak represents the ionised part and the second the non ionised part of the compound.

According to Dolan (2013) it is possible to affect the way the molecule is behaving inside the column by changing the pH of the mobile phase. More acidic eluent forces the analytes to stay in their non ionised (also non polar) form and thus retain longer in

revered phase separation (Dolan, 2013; Basniwal and Jain, 2013; Nägele and Moritz, 2005). More alkaline eluent increases ionisation and decreases interaction with the column.

The matter could be verified by trying mobile phase of different pH and see how this affects the chromatograms. If more acidic eluent produces a single peak for the analyte, the cause for peak separation was partial ionisation. Trying this is out is encouraged by other HPLC methods using a more acidic (pH=4,8/5,0) phosphate buffer as mobile phase (de Abreu et al., 2003; Phenomenex, 2014; Rao et al., 2011).

7.2 Peak shape distortions

Peak 1 did not show signs of distortions in any of the treatments. In all cases, the peak shape was ideal (Bhanot, 2014). This suggested that the experimental conditions were optimal for this compound.

Peak 2, on the other hand, behaved very differently in each treatment. Phosphate buffer had the least distortion out of the three treatments. Ammonium buffer had some, but the worst distortions were found in control. The mobile phase stayed practically the same, as did the experimental conditions.

The distortion can be due to incompatibility of the unionized form of amoxicillin (peak 2) with the current mobile phase. Change in eluent pH is not likely, as peak 1 had not been affected. Change in column temperature is as unlikely for the same reason.

Peak shape distortions may have had some effect on the integration process and the way RT is determined. If the tip of a peak is split, it gets integrated as two separate peaks. On the other hand, the middle point of a broadening peak is moving horizontally. This directly affects RT, but still fails to fully explain the quite strong fluctuations of RT_{peak2}.

7.3 Travelling peaks

Another interesting phenomenon is the travelling peak 2 in control and ammonium buffer (Figures 19 and 21). RT is decreasing in the first one and increasing in the second one. The cause for the phenomenon is unknown. Normally this suggests changes in the measurement conditions (eluent and column) but this is not the case since RT_{peak1} remains stable.

There is possibly is a link between RT fluctuations and shape distortions of peak 2. This can be noticed by comparing shape of peak 2 to the corresponding RT scatter plots. RT_{peak2} in phosphate buffer was stable but also had the least distortions.

One possible cause is change in diluents pH. As control was prepared on 2% MeOH solution, there was no buffering capacity. Gradual absorption of CO_2 from air made the solution slightly acidic. Volatilisation of ammonia may have caused something similar in ammonium buffer.

Escape of ammonia cannot be prevented, as the solution pH is one of the factors controlling its volatilisation rate (Richard, 1996). The vial had been sealed, but when injection was performed, the needle punctured the cap, thus allowing some gases to leave the vial.

In the case of control, pH could be set to pH 8,5 with NaOH. In order to get some buffering capacity, a weak buffer (5-10mM) could be used.

7.4 Peak area

As figure 24 displays, A_{total} of peak 1&2 was increasing in ammonium buffer due to rapid increase in A_{peak2}. The cause for this is unknown.

Increase in A_{peak} indicates increase in the amount of analyte. This is naturally impossible, so there must be another reason for this phenomenon. One possible

explanation is that the volatilisation of ammonia disturbed the separation process in the column, thus affecting the results.

Normally A_{total} is not a good indicator of degradation, since the degradation products of the original compound will absorb some UV radiation. Therefore concluding remarks were not done based on A_{total} values.

7.5 Degradation

As the data presented too many uncertainties, it was not possible to observe degradation of the detected compound(s). While some molecular changes might have occurred, these could not be identified due to the shape distortions and travelling peaks in the case of control and ammonium buffer.

It has been reported that "ionic strength is one of the important factors responsible for amoxicillin degradation" in alkaline solutions (Kakimoto and Funamizu, 2006; Rao et al., 2011). This had not been controlled in this experiment, which could be one of the reasons why no degradation was observed.

Another major issue is that MeOH might have been unsuitable to be used as a solvent for the stock solution of AMX. It is possible that AMX had started to degrade in the 95% MeOH solution during storage. Rao et al. (2011) states that "degradation of amoxicillin (sodium salt) at higher concentration became faster in the presence of ... alcohols". While AMX used in this experiment was trihydrate form, it still might have behaved in a similar manner in the presence of MeOH.

Appendix A displays an interesting phenomenon, where peak 2 was changing into another compound in the phosphate and ammonium buffers. This had occurred during method development. While this could not be reproduced in the actual experiment, it was an important observation.

8 CONCLUSION

In HPLC measurements two analyte peaks were detected per every injection. One of these could have been AMX and the other a degradation product. However, as no MS was performed, it was not possible to identify the compound(s).

Separate peaks might have been caused by partial ionisation of the analyte. On the other hand, it could be that AMX had started to change in the 95% MeOH stock solution, therefore having some degradation products already before spiking. This matter requires confirmation.

While peak 2 displayed molecular changes in phosphate and ammonium buffers during method development, this phenomenon could not be reproduced in the experiments.

9 POSSIBILITIES FOR FUTURE WORK

The experiments left several issues open.

Firstly, it would be interesting to know how more acidic eluent would affect the way the peaks are formed. There is also possibility for applying altogether different eluent for this method. In previous work a 10mM ammonium formate + 0.3% formic acid eluent (Ishiduka, 2013) was used in a LC/MS method to measure SMZ, AMX, OTC and TYL from a single injection using multiple channels.

The method of preparing the stock solution needs critical approach. While using the MeOH solution had its advantages, it is possible that it is unsuitable for this pharmaceutical due to instability of AMX in the solution. A weak acid or alkaline solution could be tried.

Future experiments could be performed using a single buffer solution at different strengths (for example 50mM, 100mM, 400mM). However, there is a possibility that more peak distortions will occur when using a stronger diluent solution.

Another interesting topic would be to identify the different peaks detected. This information would be beneficial in determining the cause(s) for separate peaks to appear in the chromatogram.

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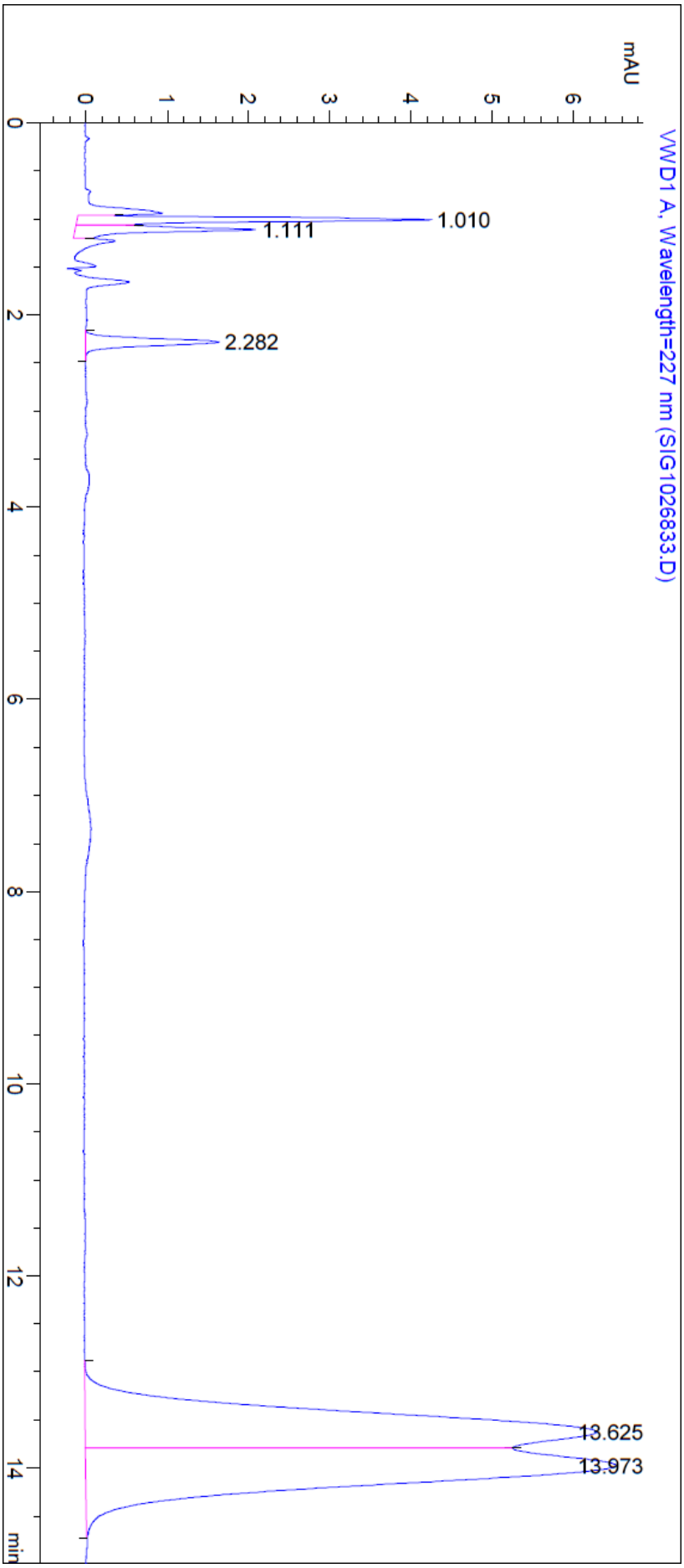
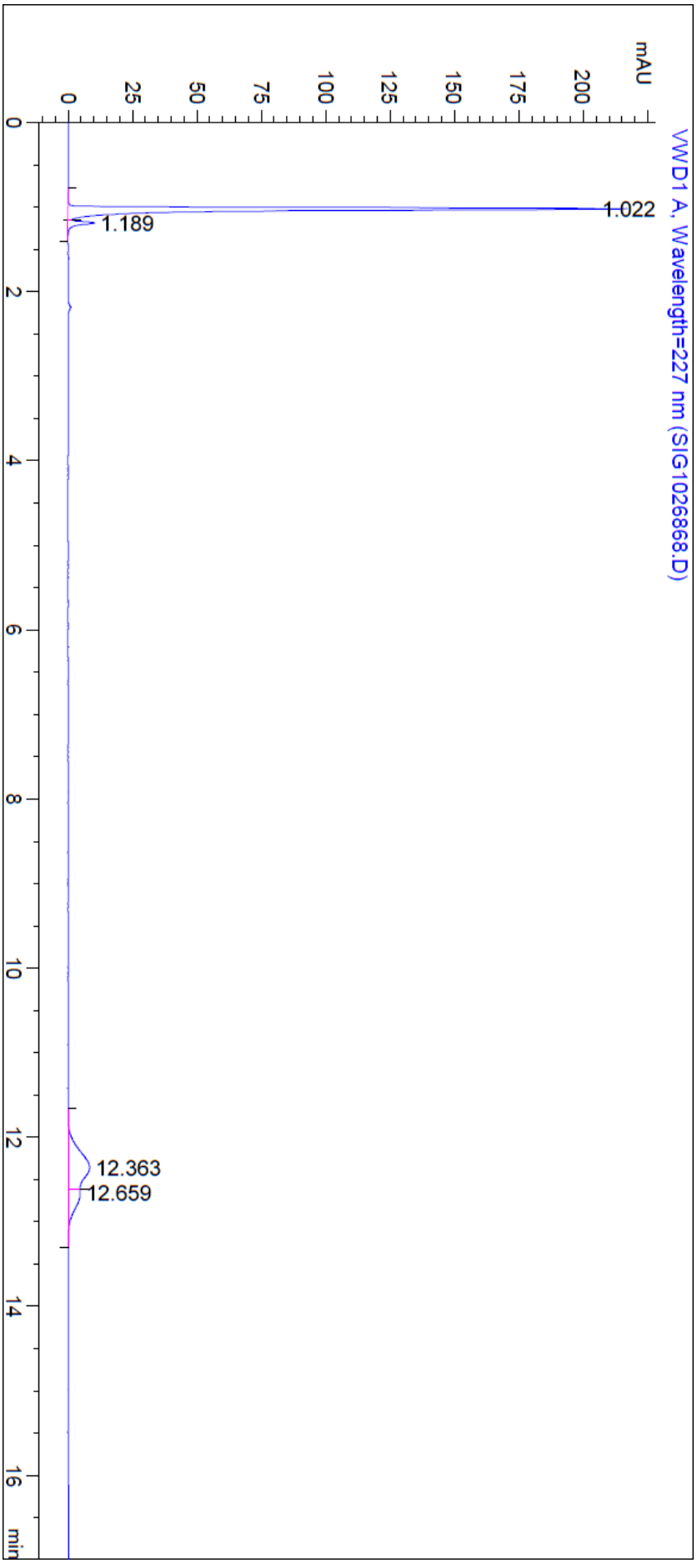
12 APPENDIX A

Chromatogram for 200mM pH 8,5 phosphate buffer+1,5% MeOH+15ppm AMX injection (45min) (SIG1026833).

Chromatogram for 200mM pH 8,5 ammonium buffer+1,5% MeOH+15ppm AMX injection (51min) (SIG1026868).


mAU
0
1
2
3
4
5
6
VWD1 A, Wavelength=227 nm (SIG1026833.D)
1.010
1.111
2.282
13.625
13.973
min
0
2
4
6
8
10
12
14


mAU
0
25
50
75
100
125
150
175
200
VWD1 A, Wavelength=227 nm (SIG1026868.D)
1.022
1.189
12.363
12.659
min
0
2
4
6
8
10
12
14
16