Sustainability in the OR: reducing environmental impact and hospital specific waste by filtration of used cardioplegic solution to exclude active pharmaceutical ingredients before disposal

Master Thesis

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Abstract

A low-budget sustainable method was developed for the filtration and quantification of procaine in used cardioplegic solution. The quantification method consisted of reversed-phase high-performance liquid chromatography with ultraviolet detection (RP-HPLC-UV) using a 5 µm XSELECT CSH C18 column and UV-detection at 294 nm. The mobile phase was recycled during measurements to minimize liquid waste, except for the last three validation sets. The HPLC method was validated for procaine in cardioplegic solution spiked with fetal bovine serum. Metronidazole was used as an internal standard. Sample preparation consisted of protein precipitation with ice cold (-20 °C) acetonitrile followed by centrifugation at 7 °C. Then samples were diluted with water to acquire the needed concentrations. Dilution was followed by a second centrifuging step (at 7 °C) to minimize serum peaks. The limit of detection (LOD) was determined to be 0.073 µg/mL. The lower limit of quantification (LLOQ) was at 0.5 µg/mL. The linearity was proven for a range of 0.5 to 500 µg/mL (r^2 = 1.0000). Accuracy and precision of the quality control (QC) samples were determined with an intra- and interday analysis. Accuracies intraday ranged from 86% to 109%, except for LLOQs of set 3 (>115%) in which mobile phase was recycled. Intraday precisions were all below 15% and for LLOQ all below 20%. The interday analysis showed that accuracies were all within limits at every QC level. The interday precisions were also all within limits at every QC level (<15% and <20% for LLOQ). The method for filtration of procaine was based on solid phase extraction (SPE) using activated carbon as a low-budget SPE material. The adsorption capacity of activated carbon for procaine in pharmaceutical solution was determined to be 74% for 100 mg of activated carbon. An estimated 1.5 grams of activated carbon would be required for filtration of one intravenous (IV) bag of cardioplegic solution. A cartridge filled with activated carbon has yet to be developed and tested on used cardioplegic solution. Ultimately, this validated method could be used as a stepping stone in order to develop a universal method for filtration of active pharmaceutical ingredients (APIs) in all kinds of perfusates.

Introduction

Cardioplegic solution is administered to the heart during cardiac surgery in order to induce reversible cardiac arrest. The solution contains several electrolytes, of which potassium chloride as the key component as it causes cardiac arrest [1]. One of the variations of cardioplegic solution contains an added active pharmaceutical ingredient (API), namely procaine [2]. According to perfusionists in Dutch hospitals, approximately 1 to 10 Liters of cardioplegic solution is used per patient. Since the solution contains APIs, the proper way of disposal would be via hospital specific waste. However, it is common practice to dispose of used cardioplegic solution via the hospital sewage system. The APIs in cardioplegic solution could potentially be harmful to the environment, specifically surface water. The main reason for disposal of the solution via the hospital sewage system is the high cost of hospital specific waste. The Catharina hospital in Eindhoven is actively involved with the chain approach pharmaceuticals in the environment, also known as 'Ketenaanpak Medicijnresten uit Water'. This is a national approach to reduce medicinal waste in the environment [3]. Another benefit of filtering cardioplegic solution before disposal is the low costs as opposed to disposal via hospital specific waste. Ultimately, it would be beneficial for the environment and in terms of the costs if a method could be developed for filtration of cardioplegic solution to remove APIs so it can be disposed of via the sewage system.

The cardioplegic solution used in the Catharina hospital in Eindhoven contains the API procaine hydrochloride. The concentration of procaine in this solution is 272 µg/mL [4]. One of the possible methods for removing APIs from a liquid is solid phase extraction (SPE). A commonly used SPE material is activated carbon, which is a relatively low cost SPE material. It is generally known that approximately 99% of a compound can be removed by filtration with activated carbon. If 99% of procaine would be removed from the cardioplegic solution, a concentration of 2.72 µg/mL would be left after filtration. The amount of procaine that would end up in surface water would be almost negligible with such low concentrations. Thus, cardioplegic solution could be disposed of via the hospital sewage system by removing procaine using activated carbon as SPE material.

In order to be able to confirm that 99% of procaine has been removed from the cardioplegic solution, a method for quantification of procaine has to be developed. The quantification of procaine in used cardioplegic solution will be conducted with reversed-phase high-performance liquid chromatography with ultraviolet detection (RP-HPLC-UV). The quantification method will be developed from scratch, after which it will be optimized. Subsequently, the optimized method will be validated based on ICH guidelines [5]. The matrix of the samples during validation will consist of cardioplegic solution spiked with fetal bovine plasma to simulate used cardioplegic solution.

In the course of this research project, the aspect of sustainability will play a key role. The mobile phase for RP-HPLC will be continuously recycled during measurements to minimize waste. All of the prepared solutions, such as blank cardioplegic solution and blank matrix, will be stored in a refrigerator after preparation and reused as much as possible during validation. Standard solutions will be prepared using a maximum volume of 50 mL to reduce liquid waste. Pipette tips will be reused as much as possible without compromising the integrity of the preparations. All of these measures will contribute to a more sustainable way of conducting experiments during this project.

The aim of this research project is to develop a method to remove procaine from used cardioplegic solution so it can be disposed of via the hospital sewage system. The extraction of procaine will be based on SPE using activated carbon as a low cost SPE material. The initial goal was to develop a cartridge filled with the amount of activated carbon that is needed to filter one intravenous (IV) bag of cardioplegic solution. The used cardioplegic solution would go through the cartridge, after which the filtered solution would be collected for disposal via the sewage system. The contents of the cartridge, containing activated carbon and procaine, would then be disposed of as solid waste. In order to obtain reliable results, the method for quantification of procaine would have to be validated. Therefore, a method for quantification of procaine in used cardioplegic solution needs to be developed and validated based on ICH guidelines [5]. To our knowledge, a method for quantification of procaine in used cardioplegic solution has not yet been validated. The determination of procaine in other matrices has been shown in several studies (Table 1). However, this will be the first validated method for quantification of procaine in used cardioplegic solution using RP-HPLC-UV, which will be a low-budget and low impact method. Ultimately, an answer has to be formed on the following research question: How effective is an SPE cartridge containing activated carbon in filtering procaine from used cardioplegic solution so it can be disposed of via the hospital sewage system?

Table 1. An overview of a selection of studies regarding the determination of procaine in different matrices using different quantification methods.

Study	Quantification	Extraction	Matrix	LoD	Linearity
Stevenson *et al.* [6]	High-performance liquid chromatography (HPLC)	Liquid-liquid extraction (LLE)	Equine plasma and urine	1 ng/mL in plasma and 10 ng/mL in urine	ND*
Luo *et al.* [7]	High-performance liquid chromatography with diode array detection (HPLC-DAD)	Liquid-liquid extraction (LLE)	Equine plasma and urine	0.01 µg/mL in plasma and in urine	0.025-25 µg/mL in plasma (r^2 = 0.9987) and 0.10-10 µg/mL in urine (r^2 = 0.9996)
Zientek *et al.* [8]	High-performance liquid chromatography with mass spectrometry (HPLC-MS-MS)	Liquid-liquid extraction (LLE)	Equine plasma	50 pg/mL	0.1-40.0 ng/mL
Qin *et al.* [9]	High-performance liquid chromatography with ultraviolet spectrometry (HPLC-UV)	Liquid-liquid extraction (LLE)	Human plasma	ND*	0.05-5.0 µg/mL ($r^2 \geq 0.998$)
Liang *et al.* [10]	High-performance liquid chromatography with ultraviolet spectrometry (HPLC-UV)	Magnetic solid phase extraction (MSPE)	Human plasma	0.004 mg/L	0.02-5.00 mg/L (r^2 = 0.9993)

*ND: Not Determined

Methods

Materials

Chemicals

Procaine hydrochloride (pharmaceutical grade) was obtained from Fagron (Capelle aan den IJssel, The Netherlands), OPG Farma (Utrecht, The Netherlands) and BUFA (IJsselstein, The Netherlands). Para-aminobenzoic acid (analytical grade), procainamide hydrochloride (analytical grade), sulfacetamide sodium (analytical grade), ranitidine hydrochloride (pharmaceutical grade) and metoclopramide hydrochloride (analytical grade) were provided by the Utrecht University Pharmaceutical Analysis Lab in the 'Koningsbergergebouw' (KBG) (Utrecht, The Netherlands). Magnesium chloride hexahydrate (analytical grade) and citric acid monohydrate (analytical grade) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Potassium chloride (analytical grade) was obtained from Chem-Lab (Zedelgem, Belgium). Sodium chloride (pharmaceutical grade) and activated carbon (pharmaceutical grade) were obtained from BUFA (IJsselstein, The Netherlands). Calcium chloride dihydrate (analytical grade) was obtained from Merck (Darmstadt, Germany). Metronidazole (pharmaceutical grade) was obtained from Genfarma (Zaandam, The Netherlands). Sodium borate buffer (0.1 M, pH 9.5), sodium borate buffer (0.1 M, pH 8.5), citric acid buffer (0.1 M, pH 6.6), citric acid buffer (0.1 M, pH 5.5), citric acid buffer (0.1 M, pH 4.4), ammonium formate buffer (0.1 M, pH 3.5), phosphoric acid (0.1 M, pH 2.5), hydrogen chloride solution (0.1 M), sodium hydroxide solution (0.1 M), sodium hydroxide solution (1 M), sodium hydroxide solution (5 M), acetic acid (100%) and perchloric acid were available as prepared solutions at the Utrecht University Pharmaceutical Analysis Lab in the KBG (Utrecht, The Netherlands). Methanol (HPLC-grade) and acetonitrile (HPLC-grade) were obtained from Biosolve (Valkenswaard, The Netherlands). Water for injections in bulk was prepared at the Utrecht University Pharmaceutical Preparations Lab in the KBG (Utrecht, The Netherlands). HyClone® Fetal bovine serum (triple 0.1µm sterile filtered research grade EU approved) was obtained from Perbio (Erembodegem, Belgium). The protein concentration of HyClone® Fetal bovine serum is unknown, but equivalent products contain approximately 35-45 mg/L. Cardioplegic solution blank concentrate was prepared by mixing sodium chloride 2.34 g, potassium chloride 4.62 g, magnesium chloride hexahydrate 15.532 g, calcium chloride dihydrate 0.147 g, and adding water for injections in bulk to obtain a 1 L solution. Blank matrix was prepared by adding 2.5 mL HyClone® Fetal bovine serum to 10 mL cardioplegic solution blank concentrate, and adding demineralized water to obtain 50 mL of solution.

Equipment

The chromatographic system consisted of components from Shimadzu (Kyoto, Japan): DGU-14A Degasser, FCV-10AL VP Pump, LC-10AD VP Liquid Chromatograph, SIL-10AD VP Auto Injector, SPD-10A VP UV-VIS Detector, CTO-10A VP Column Oven, SCL-10A VP System Controller. The column was the XSelect-3, which is a 4.6x150 mm XSELECT CSH C18 5 µm column from Waters (USA). The data was acquired and analysed using LabSolutions LC/GC acquisition and data analysis software (version 5.54 SP2). The centrifuge 5424 R from Eppendorf (Nijmegen, The Netherlands) and the VV3 vortex from VWR® (Amsterdam, The Netherlands) were used. A UV-1800 UV-spectrophotometer from Shimadzu (Kyoto, Japan) was used for UV/VIS-spectrometry.

Chromatographic conditions

The isocratic chromatographic measurements were conducted on the XSelect-3 column at room temperature. The mobile phase consisted of 10% v/v methanol with an addition of 10 mM sodium citrate buffer pH 3.0. An injection volume of 5 µL was applied. The flow rate was 1 mL/min. The UV/VIS detector was set at a wavelength of 294 nm. Before measurement of the validation sets, the column was rinsed with 30% methanol, then with 100% methanol and then again with 30% methanol.

Validation

Stock solutions of procaine (5 mg/mL) for the calibration line were prepared by dissolving 57.7 mg procaine hydrochloride in 10 mL demineralized water. The rest of the calibration line stocks at different concentrations were prepared by a dilution series using the initial stock solution of procaine (5 mg/mL) and demineralized water. The stock solutions for the calibration line had concentrations of 5, 1, 0.5, 0.1, 0.05, 0.01 and 0.005 mg/mL procaine in demineralized water. Internal standard solutions were prepared by dissolving 100 mg metronidazole in 25 mL acetonitrile. The calibration line standards had concentrations of 500, 100, 50, 10, 5, 1 and 0.5 µg/mL procaine in matrix. Additionally, a zero and double blank was included in every calibration line. For every validation set, two separate calibration lines were prepared. The calibration line standards were prepared by mixing 450 µL blank matrix, 50 µL internal standard solution (or 50 µL acetonitrile for the double blank) and 50 µL of the associated procaine stock solution (or demineralized water for double blank and zero). Protein precipitation was conducted by adding 700 µL ice cold (-20 °C) acetonitrile to the calibration line standards, followed by immediately mixing on a vortex. The samples were centrifuged at 15000 Revolutions Per Minute (RPM) for 10 minutes at 7 °C. The dilution of the standards was conducted by transferring 250 µL to a new polypropylene microtube and adding 750 µL demineralized water, followed by mixing using a vortex. After dilution, the samples were centrifuged again at 15000 RPM for 10 minutes at 7 °C. A volume of 200 µL of each sample was transferred to a vial containing an insert.

The Quality Control (QC) samples were prepared from stock solutions of procaine (5 mg/mL), which were also prepared by dissolving 57.7 mg procaine hydrochloride in 10 mL demineralized water. A total of 5 stock solutions of 5 mg/mL procaine were prepared per validation set, from which 5 series of QC samples were prepared. Each stock solution of procaine (5 mg/mL) was diluted into stock solutions of 2.5, 0.25, 0.025 and 0.005 mg/mL procaine in demineralized water. The internal standard solution used in the calibration line standards was also used in the QC samples. The QC samples had concentrations of 500, 250, 25, 2.5 and 0.5 µg/mL procaine in matrix. These samples were prepared by mixing 450 µL blank matrix, 50 µL internal standard solution and 50 µL of the associated procaine stock solution. Subsequently, 700 µL ice cold (-20 °C) acetonitrile was added to the samples for protein precipitation. This was followed by immediately mixing on a vortex. Then the samples were centrifuged at 15000 RPM for 10 minutes at 7 °C. A volume of 250 µL was transferred to a new polypropylene microtube and 750 µL demineralized water was added in order to dilute the samples. The samples were centrifuged a second time at 15000 RPM for 10 minutes at 7 °C. Of each sample, 200 µL was transferred to a vial containing an insert.

All of the procaine hydrochloride solutions, for the calibration lines as well as the QC samples, and the internal standard solutions were freshly prepared on the day of validation. Blank matrix solutions were stored in the freezer after preparation and reused on multiple days of validation to reduce waste. A total of 6 sets of validation were conducted during this research project. The first

couple of sets were meant as a test run. During the first three sets the mobile phase (batch of 500 mL) was continuously recycled during measurements. At the time of measurement of validation set 3, the recycled mobile phase had already been excessively polluted due to the previous sets of measurements. A fresh and clean batch of mobile phase (batch of 2 L) was used on validation sets 4, 5 and 6 without recycling during measurements in order to acquire data without the influence of using recycled mobile phase.

Results and discussion

The molecular structure of procaine contains an aromatic amine group, which functions as a chromophore (Figure 1). It also contains an ester group in the middle of the structure [11]. Procaine has a functional group with a pKa of 9, likely belonging to the tertiary amine group [12]. The functional group with a pKa of 9 will be positively charged at a pH of 7 and lower. In the world of pharmaceutical analysis, a commonly used method for RP-HPLC consists of a mobile phase with a certain percentage of methanol with the addition of perchloric acid. Since this is a proven method, it was chosen as a starting point during this project. The addition of perchloric acid causes the mobile phase to have a pH of 2.3. At this pH, the amine group with a pKa of 9 will be positively charged. It is to be expected that procaine will have little to no retention due to the positive charge and will likely elute in the dead time. However, the added perchloric acid in the mobile phase will act as a weak ion pair with the positively charged amine group of procaine. This ion pair interaction will shield the positive charge on procaine, which will make it less likely for procaine to interact with water molecules in the mobile phase. As a result, procaine will be pushed towards the stationary phase and will therefore have more retention. The ability of perchloric acid to act as an ion pair with positively charged functional groups is the main reason behind the success of this method. This is a solid method for both acidic as well as basic compounds, seeing that perchloric acid will cause acidic compounds to be neutral and will act as an ion pair for basic compounds. For this method, the specific extinction (A11) and its associated maximum wavelength (λ_{max}) of procaine in acidic environment had to be applied. According to literature, the λ_{max} of procaine in acidic environment was 294 nm. However, there was no A11 in acidic environment reported. The A11 of procaine measured in methanol was chosen instead, which was 954 at 296 nm [12]. Since the chromophore is relatively distant from the tertiary amine that is affected by pH variation, the assumption was made that there would not be a difference in absorption between the charged an uncharged forms of procaine.

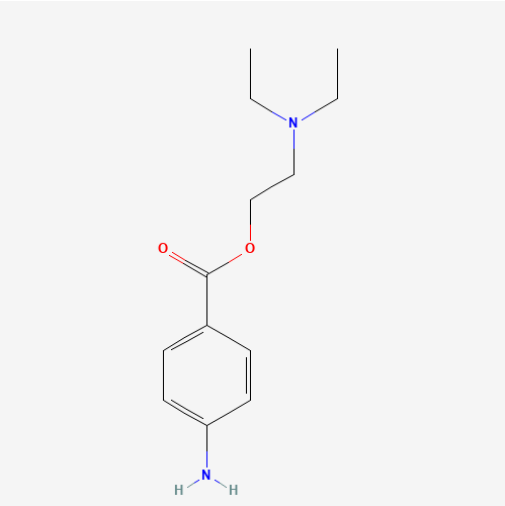

Figure 1. Structure of procaine [11].

The initial method was based on the database from the KBG Lab at Utrecht University, which contained retention factors associated with commonly used mobile phases and columns. The initial method consisted of 10% w/w methanol with 10 mM $HClO_4$ as the mobile phase with the RS6 column on a flow rate of 0.5 mL/min and injection volume of 5 µL measured at 294 nm (RP-HPLC Method 1). The first standard of procaine hydrochloride in demineralized water had a concentration of 23.7 µg/mL, calculated using an A11

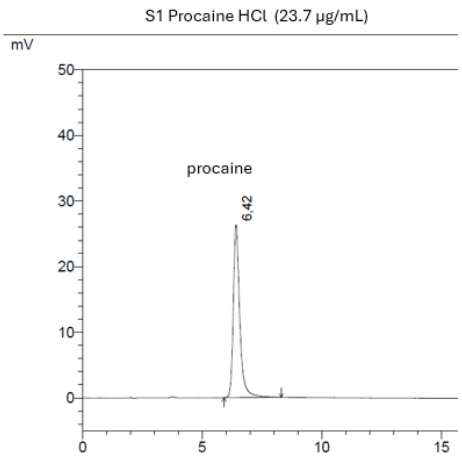

Figure 2. Chromatogram of procaine HCl standard 1 with a concentration of 23.7 µg/mL. Method: 10% w/w MeOH + 10 mM HClO4, RS6 column, flow rate 0.5 mL/min, injection volume 5 µL, 294 nm.

of 954 and the rule of thumb of 20/A11. This is a rule of thumb that can be applied with the systems in the KBG Lab. According to this rule of thumb, a solution with this concentration should result in a peak of approximately 100 mV when the retention factor is equal to 1. However, the results showed a peak of procaine of approximately 30 mV (Figure 2). Since the peak was distinctly lower than expected, the cause for the lower absorption had to be determined.

The first avenue explored was the stability of procaine. As previously mentioned, procaine contains an ester group. The hydrolysis of this ester group results in the formation of para-aminobenzoic acid (PABA) [13]. In theory, the decomposition of procaine into PABA could lead to a lower main peak of procaine. The presence of a chromophore in PABA makes it detectable by UV. Therefore, a second peak would be present in the chromatogram when decomposition has occurred. The standard from the previous experiment (standard 1) was measured again after 5 days and two new standards (standard 2 and 3) were prepared and measured. Additionally, a sample of PABA in demineralized water was prepared and measured. The retention time of PABA was determined to be 3.52 minutes (Figure 3). The results showed that standard 1 that was kept in the vial inside the HPLC now only had a peak at 3.53 minutes, which is exactly at the retention time of PABA (Figure 4a). Standard 1 that was kept in the volumetric flask on the desk did not show a peak around 3.52 minutes (Figure 4b). It seemed that procaine was not stable inside the vial in a dark environment, but was stable when exposed to light. Standards 2 and 3 did not show a second peak on the day of preparation.

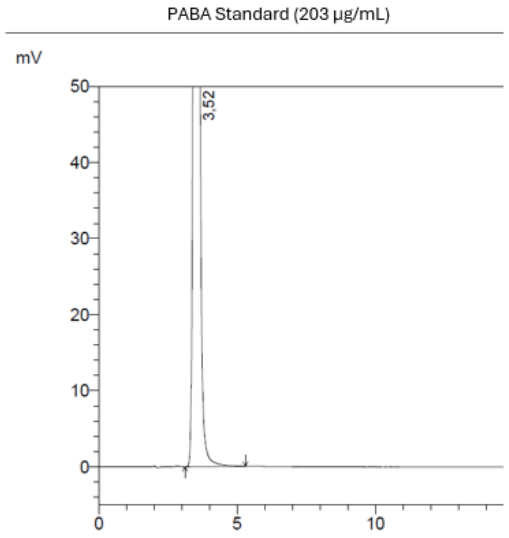

Figure 3. Chromatogram of PABA with a concentration of 203 µg/mL. It is shown that PABA has a retention time of 3.52 minutes.

In a follow-up experiment, all of the standards were measured again after two days. The chromatograms of standards 2 and 3 that were kept in the dark as well as those kept in the light now showed a small second peak at around 3.52 minutes (Figure 4c). Standard 1 that was still kept in the vial now had no peaks in the chromatogram. However, standard 1 that was kept in the volumetric flask on the desk in the light showed no second peak. The results regarding the stability of standard 1 could not be explained and were labelled as an artefact, seeing that no other standard solution was giving similar results. The other standards did not show significant decomposition after two days. In order to further investigate if decomposition of procaine is stimulated in a dark environment, standard 3 was measured after 2 weeks of being kept in the volumetric flask wrapped in aluminium foil and the results were similar to the previous measurements of this standard solution (Figure 4d). Moreover, it was investigated if the lower peak was being caused by decomposition of procaine in an acidic environment. A solution of procaine in 0.1 M HCl was measured (RP-HPLC Method 1 with RS24 column) and no second peak was present in the chromatogram. The results of the stability experiments showed that decomposition of procaine does occur over time. However, the peak of PABA seemed to only be approximately 1% of the main peak of procaine. Thus, the significantly lower peak of procaine cannot be explained by the occurrence of decomposition into PABA.

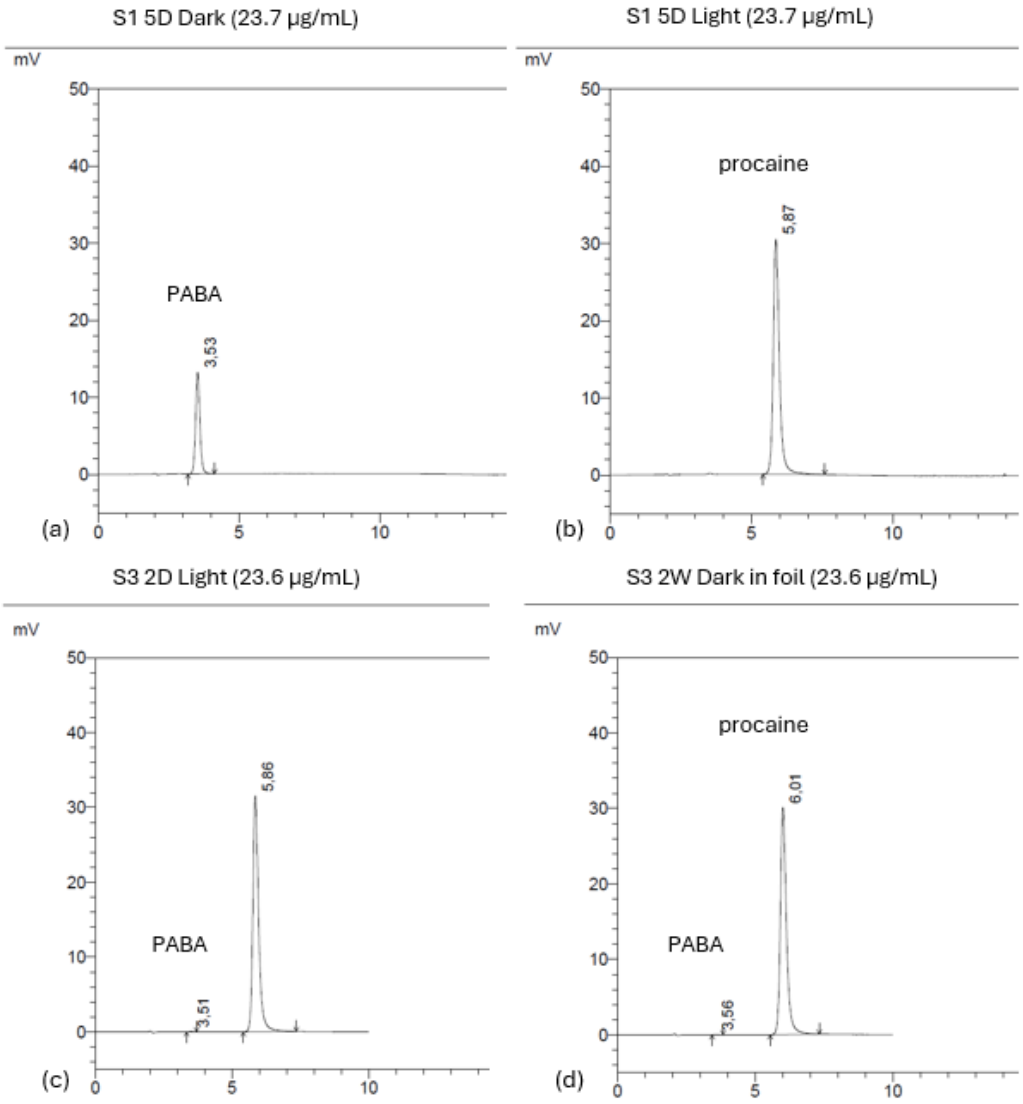

Figure 4. (a) Chromatogram of standard 1 after 5 days in the dark. It shows a single peak at 3.53 minutes, which is at the retention time of PABA. (b) Chromatogram of standard 1 after 5 days in the light. It shows a single peak at 5.87 minutes, which is around the retention time of procaine. (c) Chromatogram of standard 3 after 2 days in the light. It shows a second peak at 3.51 minutes, which is at the retention time of PABA. (d) Chromatogram of standard 3 after 2 weeks of being kept in a volumetric flask covered with aluminium foil.

Another possible cause for the lower peak of procaine could be a lower absorbance due to the occurrence of a pH shift. As previously mentioned, data found in literature was used for setting up the initial method. It was found that procaine seemingly only has one functional group with a pKa, namely a pKa of 9 belonging to the tertiary amine group [12]. No other pKa was reported. However, procaine does also contain an aniline group, which in theory has a pKa of 4.6. The aniline group would also be positively charged at a pH of 2.3, which is the pH of the mobile phase in the initial method. Since the aniline group is directly attached to an ester group, the pKa would probably be even lower due to and inductive effect. Moreover, this aniline group is part of the chromophore which in theory could influence the absorbance of the molecule. In order to determine if a pH shift occurs at a certain pH, a series of solutions of procaine in buffers with varying pH were measured with UV/VIS-spectrometry. The UV-spectra of procaine in the higher pH range, from pH 3.5 to 9.5, did not show a shift in absorbance. At pH 2.5, a slight shift was observed (Figure 5a). Therefore, an extra set of solutions was measured in the lower pH range, from pH 2.5 to 1. As an additional

measurement, a solution of procaine in the mobile phase used in the initial HPLC method was measured. The results showed that procaine has a pH shift at pH 2.5 (Figure 5b). Moreover, this experimental finding was consistent with UV-spectrometric data found in literature of PABA. According to literature, the A11 of PABA in alkalic conditions is 1063. However, the A11 in acidic conditions drastically decreases to 95 [14]. The drastic decrease in A11 in acidic environment of PABA can be extrapolated to procaine, seeing that PABA is the chromophoric group in the structure of procaine. It can be further confirmed that the pH shift of PABA, and therefore also of procaine, is caused by the protonation of the aromatic amine group. The structure of PABA contains two functional groups that could be affected by a change in pH, which is the aromatic amine group and the carboxylic acid group. The aromatic amine group in PABA is also known as an aniline. The UV-spectrometric data of aniline found in literature shows the same phenomenon as PABA, in which the absorbance drastically lowers in acidic environment [15]. In conclusion, the aniline group in procaine causes a pH shift at acidic environment which leads to a drastically lower absorbance. It was decided that the mobile phase had to be changed in order to measure outside of the pH shift of procaine. Thus, a mobile phase with a pH of at least 2.5 had to be used for the rest of the project.

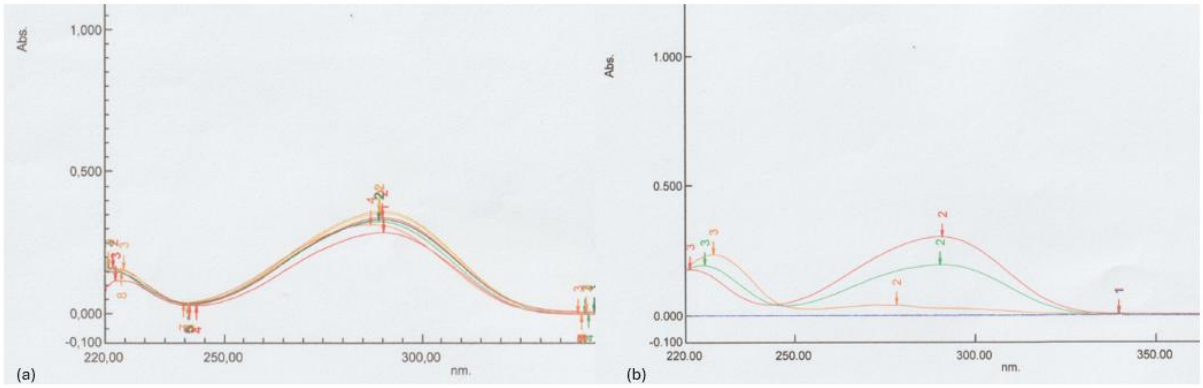

Figure 5. (a) UV-spectra of procaine solutions in buffers with varying pH from 3.5 to 9.5. It shows a shift in absorbance at pH 2.5. (b) UV-spectra of procaine solutions in buffers with varying pH. The top line is at pH 2.5, the middle line is at pH 2.3 (mobile phase), the lower line is at pH 1.

A calibration line of procaine was made to determine the linearity. The previous chromatograms showed that standard procaine solutions of approximately 21 µg/mL resulted in peaks of approximately 30 mV. It is known that HPLC measurements up to 1000 mV often still have linearity. Therefore, a concentration of 600 µg/mL should produce a peak of approximately 1000 mV. One of the previous chromatograms showed a peak of 0.071 mV to still be at least three times greater than the noise. Based on the peaks of the procaine solutions with a concentration of 21 µg/mL, it can be calculated that a concentration of 0.050 µg/mL will probably still be detectable. The concentration range of the calibration line was chosen based on these estimates, in which the highest point was 600 µg/mL and the lowest point 0.050 µg/mL. A dilution series was conducted using a dilution factor of 2 to prepare the calibration standards. The calibration line was measured using RP-HPLC Method 1 (RS6 column, 10% w/w methanol with 10 mM $HClO_4$, flow rate 0.5 mL/min, injection volume 5 µL, 294 nm). The data was analysed by conducting a normal linear regression and it resulted in $R^2 = 1.0000$ (Figure 6). This result supports that there is a linear correlation between the response of the HPLC (area) and the concentration of procaine. Since the calibration line was measured with the mobile phase with pH2, it has to be noted that it was measured at a pH shift.

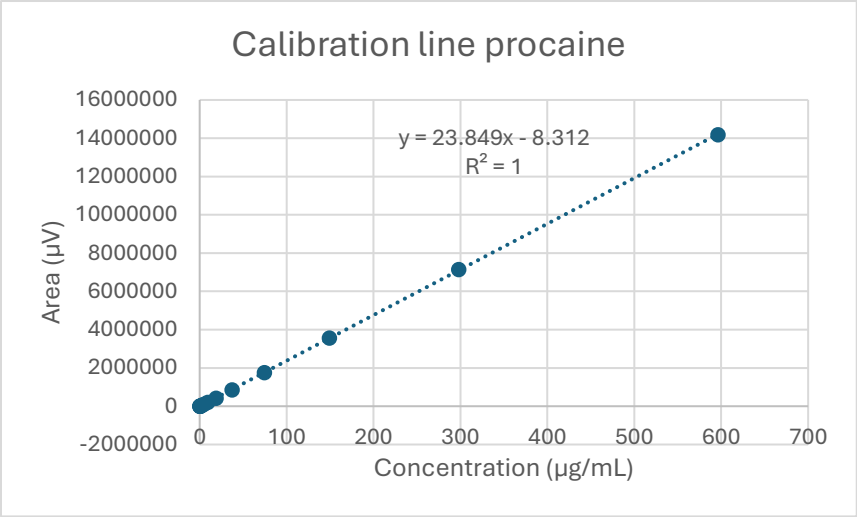

Figure 6. Calibration line of procaine in demineralized water measured with RP-HPLC Method 1.

Optimization

The optimization of the initial RP-HPLC method was initiated by changing the mobile phase to 15% v/v methanol with 15 mM sodium acetate buffer pH 4.8. In theory, the application of this mobile phase should lead to a peak of around 100 mV as the pH shift would be avoided. Unfortunately, this resulted in a peak with excessive tailing with a tailing factor of 3.67 (Figure 7a). A few different columns were tested in combination with this mobile phase and the best peak was obtained with the XSelect3 column (Figure 7b).

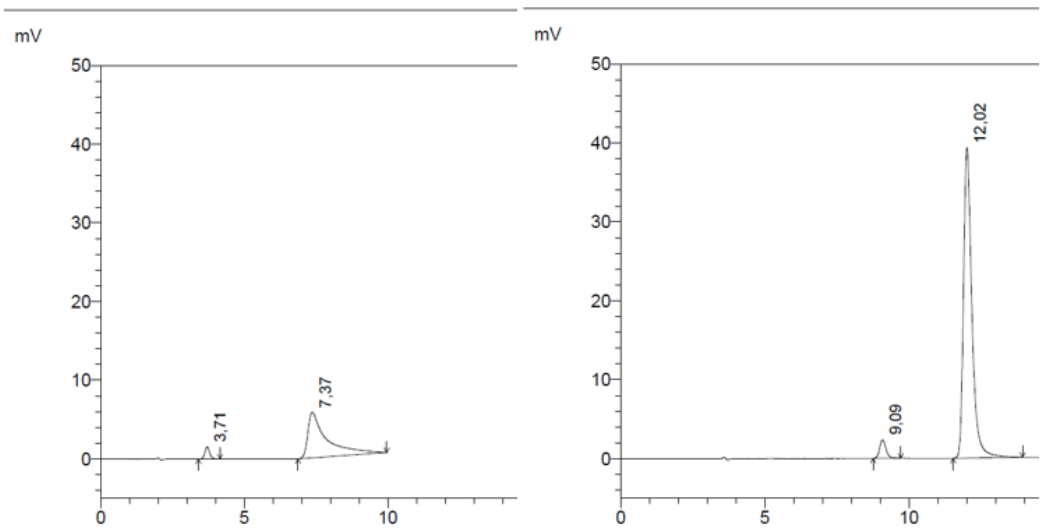

Figure 7. (a) Chromatogram of procaine standard 4 measured with RP-HPLC Method 1 with a changed mobile phase of 15% v/v methanol with 15 mM sodium acetate buffer pH 4.8. (b) Chromatogram of procaine standard 3 (kept in aluminium foil for 2 weeks) with mobile phase of 15% v/v methanol with 15 mM sodium acetate buffer pH 4.8 in combination with XSelect3 column.

Although the peaks that were obtained with the new mobile phase were acceptable, it was decided to change to another mobile phase. It would be beneficial in the case of future measurements of other compounds to have a mobile phase with a pH lower than 4.8, since most acidic compounds would be more than 50% charged at pH 4.8. As previously mentioned, the pH shift of procaine was at pH 2.5. Therefore, the most ideal pH for the mobile phase would be at pH 3. The XSelect3 column in combination with 15% v/v methanol with 10 mM sodium citrate buffer pH 3 resulted in acceptable peaks for both procaine and PABA (tailing factor 1.20 and 1.15, respectively). It should be noted that on this method, procaine eluted faster than PABA due to the change in mobile phase. On a mobile phase with pH 3, PABA is neutral and procaine has a positively charged tertiary amine group. Naturally, procaine will have less retention due to its positively charged functional group as opposed to PABA. Furthermore, there was room for a faster elution, since the last peak eluted at 13 minutes. The flow rate was then changed from 0.5 mL/min to 1 mL/min. At a flow rate of 1 mL/min, the retention time of both procaine and PABA were under 10 minutes (retention time 3.95 and 6.43, respectively) (Figure 8). Ultimately, the most suitable method was 15% v/v methanol with 10 mM sodium citrate buffer pH 3 as the mobile phase in combination with the XSelect3 column on a flow rate of 1 mL/min and injection volume of 5 µL measured at 294 nm (RP-HPLC Method 2). From the moment of selecting RP-HPLC Method 2, the mobile phase was continuously recycled during measurements unless mentioned otherwise.

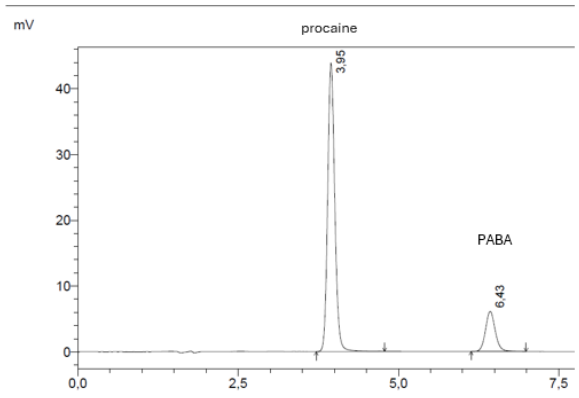

Figure 8. Chromatogram of procaine standard 4 measured with XSelect3 column and 15% v/v methanol with 10 mM sodium citrate buffer pH 3 on a flow rate of 1 mL/min.

The limit of detection (LoD) of procaine was determined using the standard with the lowest concentration of the previously prepared calibration line standards (Cal_{P14}). This standard had to be diluted until there was no detectable peak showing in the chromatograms. The measurements were made with RP-HPLC Method 2. It was estimated that the peak in the chromatogram of Cal_{P14}, was too low. An additional measurement was made of a previous standard (Cal_{P13}). The chromatograms of Cal_{P15} and Cal_{P16} showed no detectable peak. In the chromatogram of Cal_{P14}, the height of the noise was estimated to be 0.023 mV (Figure 9). Since the LoD is the concentration of a peak that is three times the height of the noise, the peak of procaine would have to be 0.069 mV. The chromatogram of Cal_{P14} showed a peak of approximately 0.07 mV and

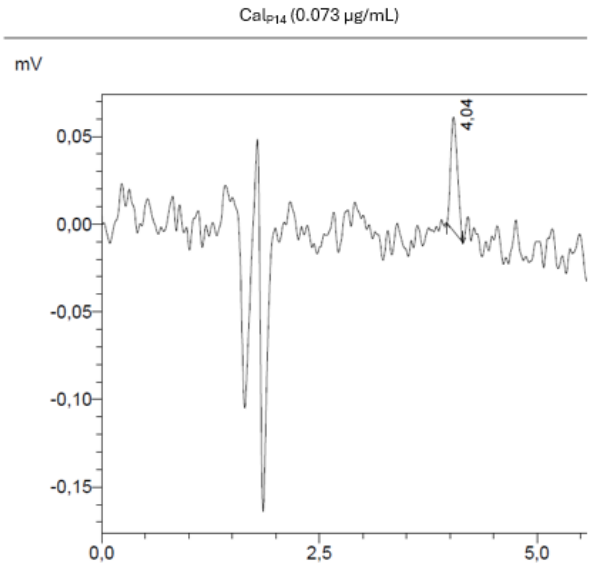

Figure 9. Chromatogram of Cal_{P14}, which is the standard of the calibration line with lowest concentration. It shows a peak of approximately 0.07 mV, which was determined to be the LoD.

the concentration of this standard was 0.073 µg/mL. Thus, the LoD of procaine is at 0.073 µg/mL with a peak of 0.07 mV.

A new calibration line was prepared and measured with RP-HPLC Method 2. The concentration range was from 0.05 µg/mL to 1000 µg/mL. A normal linear regression (R^2 = 0.9996) suggested that it is highly likely that there is a linear correlation between the response of the HPLC (area) and the concentration of procaine (Figure 10).

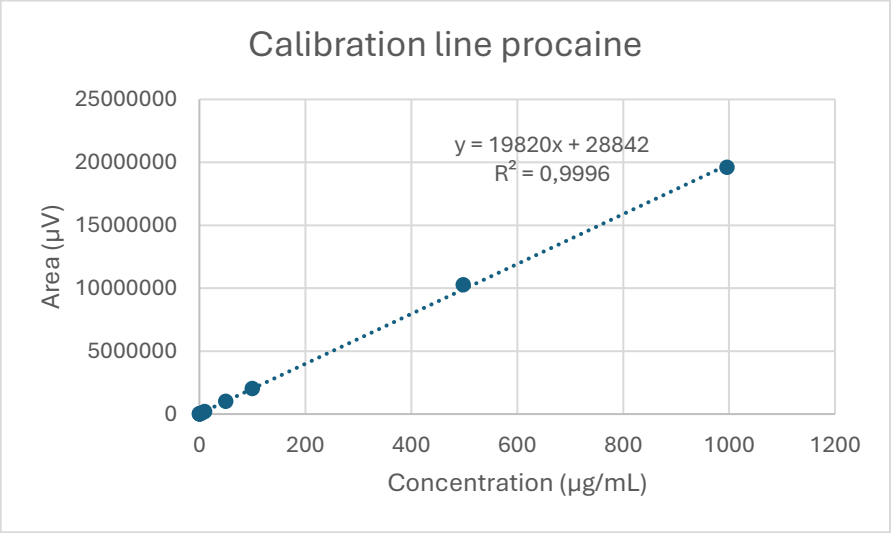

Figure 10. Calibration line of procaine in demineralized water measured with RP-HPLC Method 2.

Furthermore, an internal standard was added to the method at this point during the project. An internal standard was a necessary addition to the method, seeing that the sample preparation during the validation is complex. The preferred internal standard was procainamide, because of its similar structure to procaine. Therefore, it is assumed that procainamide and procaine will act similarly during sample preparation. A measurement of procainamide was conducted using RP-HPLC Method 2. Procainamide eluted at 2.55 minutes, which was close to the dead time of approximately 2 minutes (Figure 11). Nonetheless, it was chosen as the internal standard for it was sufficiently separated from procaine (retention time 4.10 minutes).

Prevalidation

The prevalidation was initially conducted using the HPLC method that had been developed in the previous months (RP-HPLC Method 2). The matrix of the samples was going to consist of blank cardioplegic solution spiked with fetal bovine serum. The samples would be spiked with a certain concentration of procaine in order to prepare the calibration line, except for the double blank and the zero samples. An internal standard was added to all of the samples, except for the double blank. The internal standard was a solution of procainamide in acetonitrile. The sample preparation was based on a protein precipitation using ice cold acetonitrile (-20 °C) followed by centrifuging at maximum RPM for 20 minutes. The extraction was then carried out by evaporating the liquid (at 10 mbar and 80 °C) and reconstituting the pellet in 15% methanol. During the prevalidation, a few obstacles within the method were discovered.

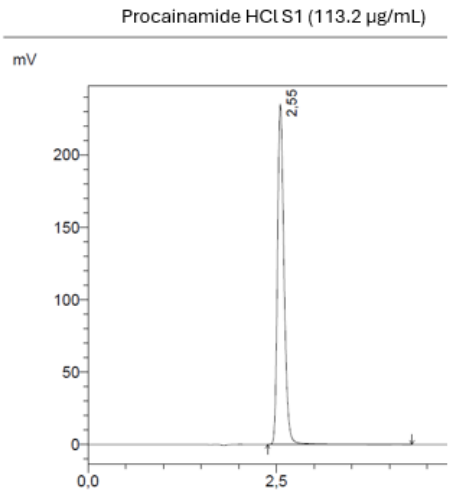

Figure 11. Chromatogram of procainamide standard 1, showing a retention time of 2.55 minutes.

An obstacle that arose was that procainamide eluted at a retention time at which serum peaks also eluted. This obstacle was going to be combatted by changing the percentage of methanol in the mobile phase from 15% to 10% (with 10 mM sodium citrate buffer pH 3), which would increase the retention time of procainamide. As a result, procainamide would elute after serum peaks. Another step that was taken to improve the peak of procainamide, was to attempt to reduce the serum peaks. The centrifugation time after precipitation was changed to 30 minutes at maximum RPM and at a lower temperature of 7 °C in order to optimize extraction. Furthermore, this centrifugation step was added after reconstitution of the pellet. Nonetheless, the internal standard procainamide kept eluting simultaneously with serum peaks (Figure 12). An extraction in water of the calibration line was conducted to determine if procainamide was actually eluting simultaneously with serum peaks. It was confirmed that procainamide eluted at the same retention time as serum peaks. A different compound had to be selected as the internal standard.

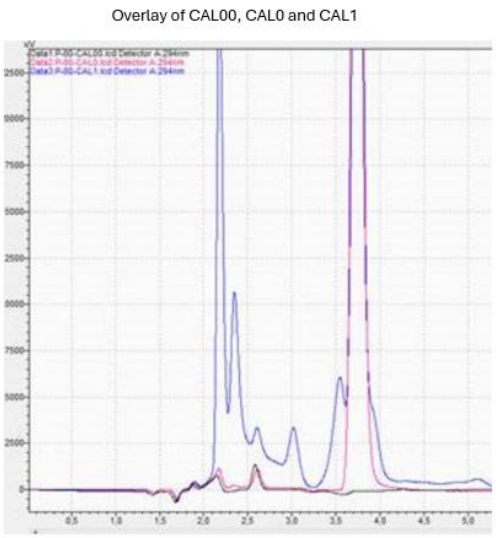

Figure 12. Overlay of chromatograms of sample CAL00, CAL0 and CAL1 from one of the calibration lines during prevalidation. It shows serum peaks at 2 to 4 minutes, which is at the retention time of procainamide (around 4 minutes). Measured with mobile phase 10% methanol + 10 mM sodium citrate buffer pH 3.

In the search for a new internal standard, the following criteria were considered. The compound has to elute on a mobile phase of 10% methanol, it has to have UV absorption at 294 nm, it has to be chemically stable and preferably not have a pKa between 1 and 5. A few compounds were selected from the database with retention factors and were measured on the current HPLC Method, including sulfacetamide sodium, ranitidine hydrochloride, metronidazole and metoclopramide hydrochloride. Sulfacetamide sodium and ranitidine hydrochloride both had retention times of around 6.5 minutes, which would overlap with PABA. On top of that, ranitidine showed many extra peaks which makes it even more unsuitable. Metoclopramide was also unsuitable, since it had a retention time of around 23 minutes. It was preferred to keep the retention times of all compounds under 10 minutes. The most suitable choice was metronidazole with a retention time of around 8 minutes, seeing that it would be sufficiently separated from procaine and PABA (Figure 13). Moreover, it would not elute at serum peaks. The selectivity of the method was hereby confirmed, since it was proven that the analyte (procaine) can be differentiated from the internal standard (metronidazole) and the matrix (serum peaks). The specificity of the method had already been proven by showing that the analyte (procaine) can ben differentiated from its metabolite (PABA).

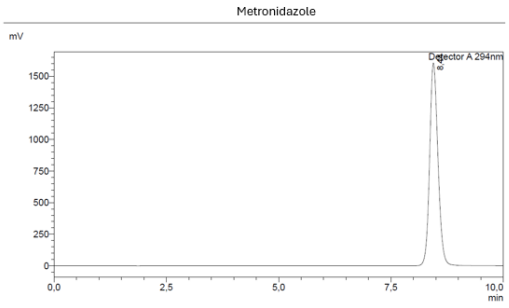

Figure 13. Chromatogram of metronidazole, showing a retention time of 8.44 minutes.

Another obstacle was that the evaporation process using a concentrator took more than 4 hours to finish. A few different evaporation methods were tested to attempt to find a method that is quick as well as effective. The methods included evaporation under nitrogen at 80 °C, evaporation under nitrogen at 100 °C and evaporation under compressed air stream at 80 °C. All of the evaporation methods resulted in chromatograms with multiple extra peaks. These unknown peaks showed up more frequently as more measurements were conducted, indicating build-up of pollution in the column. It was assumed that these extra peaks could be the result of pollution due to the blank matrix, procaine or procainamide. The extraction in water that was mentioned before, was also conducted with pure procaine and pure procainamide. The results did show that there were peaks as a result of degradation or impurities of both procaine as well as procainamide. One of the peaks was identified as PABA in a follow-up experiment (retention time 8.89 minutes). The rest of the extra peaks were assumed to be pollution due to the blank matrix.

Since the extraction method of evaporation followed by reconstitution of the pellet kept resulting in an excess amount of serum peaks, an alternative method for sample clean-up had to be selected. The benefit of the concentration method was that samples with very low concentrations could be prepared. However, it was determined that such low concentrations were not necessary to include in the validation method for this project. It was decided that approximately 99% of procaine would have to be removed from cardioplegic solution for it to be successfully filtered. This corresponds to a concentration of 2.72 µg/mL of procaine left in cardioplegic solution after filtration. Therefore, the LOQ would be 2.72 µg/mL. In the protocol using the concentration method, samples with even lower concentrations were included. It was decided that a dilution method would suffice for the current validation method. An added benefit of using dilution as the method for sample preparation would be that the proteins in the samples would also be diluted. The calibration line was altered by removing the two lowest concentrations, since those concentrations were lower than needed. The calibration line was changed from 0.001-500 µg/mL to 0.5-500 µg/mL. The optimized sample preparation consisted of a protein precipitation with ice cold acetonitrile (-20 °C) followed by centrifuging at maximum RPM for 30 minutes at 7 °C. Then the samples were diluted with demineralized water instead of concentrated by means of evaporation. After dilution, the samples were centrifuged again at maximum RPM for 30 minutes at 7 °C. A new container of procaine hydrochloride was acquired and used to spike the calibration line samples, after testing and confirming the purity of the compound. The optimized protocol, containing an alternative sample preparation and adjusted calibration line, was carried out using metronidazole as the internal standard.

Although multiple adjustments were made to the protocol to improve the quality of the peaks, there still was interference by unknown peaks. Even after improvement of sample clean-up, the samples still would contain a certain amount of protein. Logically, build-up of protein in the column over time could have been occurring. Another issue that occurred was the raising of the baseline as more samples were measured. The culprit was most likely the recycling of mobile phase during measurements throughout the optimization and

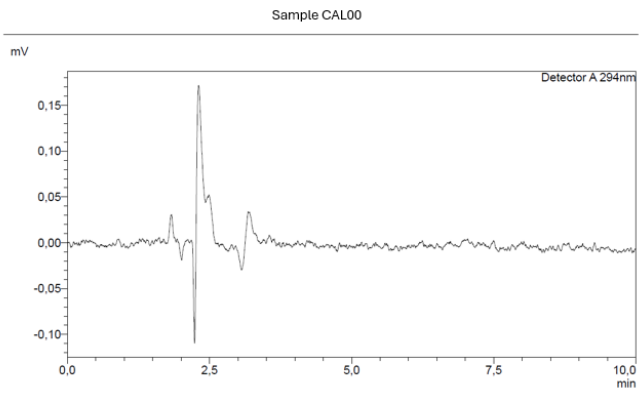

Figure 14. Chromatogram of sample CAL00, measured after rinsing the column with 100% methanol and using a fresh batch of mobile phase.

prevalidation. A bottle of 500 mL mobile phase was used during these measurements, which is a relatively small volume. In such a small volume, pollution will build-up in a short amount of time when large batches of samples are being measured. These issues were combatted by rinsing the column with 100% methanol and using a fresh bottle of mobile phase for the upcoming measurements. Subsequently, a new calibration line was prepared and measured. The baseline of the first double blank sample showed to be completely clean (Figure 14). The rest of the calibration line samples showed no extra peaks. Following the first calibration line, a sample of pure acetonitrile was injected twice in a row. Both chromatograms showed multiple unknown peaks (Figure 15). Since acetonitrile can elute almost any compound, these measurements indicated build-up in the column. It was confirmed that it is necessary to rinse the column after measurement of a large batch of samples to prevent interference by extra peaks due to build-up in the column.

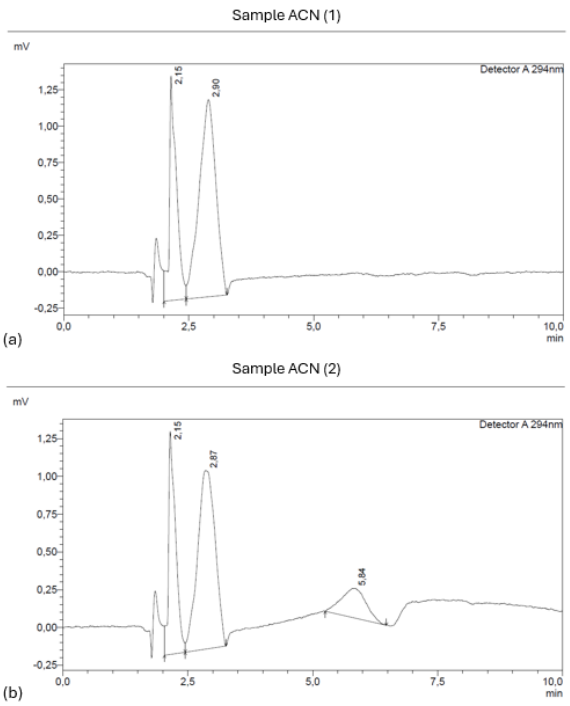

Figure 15. (a) Chromatogram of the first injection of acetonitrile after measurements of calibration line samples. (b) Chromatogram of the second injection of acetonitrile.

All of the above mentioned measurements during prevalidation were conducted using RP-HPLC Method 3 (10% v/v methanol with 10 mM sodium citrate buffer pH 3 as the mobile phase, XSelect3 column, flow rate of 1 mL/min, injection volume of 5 µL, measured at 294 nm), except for the first measurement of the calibration line at the start of prevalidation. The validation was also conducted using RP-HPLC Method 3.

Validation

In this research project a total of 6 validation sets were measured. The sets were prepared using the protocols for calibration line samples and QC samples (Appendices A and B). The first couple of sets were conducted as test runs, in order to see if the method needed optimizing. Therefore, the data of the first two sets were not analysed. The data of sets 3 to 6 were analysed and will be discussed. The following validation parameters were chosen to be determined: Lower Limit of Quantification (LLOQ), calibration line (including carry-over), selectivity, accuracy and precision, matrix effect and stability.

The Lower Limit of Quantification (LLOQ) is often chosen as the concentration that is 5 times the noise. In this project, the concentration chosen as the LLOQ was 0.5 µg/mL. The LLOQ was estimated to be approximately 16 times the noise (Figure 16). The reason behind the choice for this concentration for the LLOQ was that it would not be necessary to measure below 0.5 µg/mL, seeing that the lowest concentration of procaine in cardioplegic solution that would have to be measured would be 2.72 µg/mL.

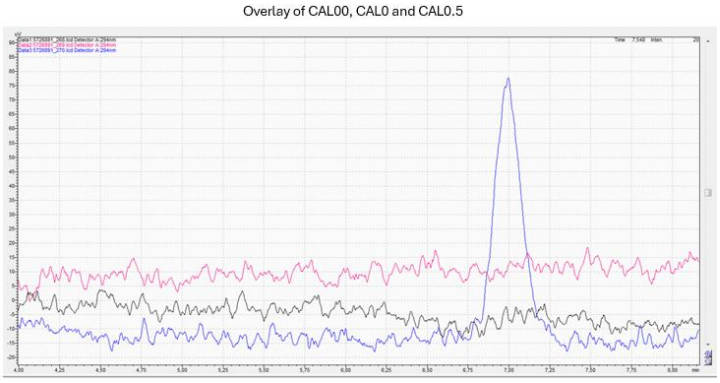

Figure 16. Overlay of chromatograms of CAL00 (black), CAL0 (pink) and CAL0.5 (blue) of validation set 4. The peak of procaine is seen at 7 minutes. It shows the peak to be approximately 16 times the noise.

The range of the calibration line was selected based on the concentrations of procaine in one IV bag of cardioplegic solution before and after filtration. The concentration before filtration would be 272 µg/mL (equal to 100%) and after filtration 2.72 µg/mL (equal to 1%). The lower limit was selected to be 0.5 µg/mL and the upper limit 500 µg/mL. The calibration line consisted of the following points: 0.5, 1, 5, 10, 50, 100 and 500 µg/mL. An overlay of the calibration line samples of set 4 is depicted in Figure 17. The overlay confirms the selectivity, seeing that procaine and metronidazole (internal standard) are sufficiently separated. In each validation set, two separate calibration lines were prepared and measured. The chromatograms of the calibration lines of set 3 showed negative peaks in the baseline at the

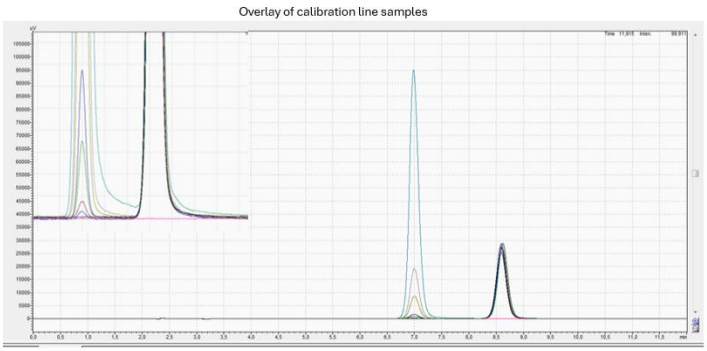

Figure 17. Overlay of chromatograms of the calibration line samples of set 4.

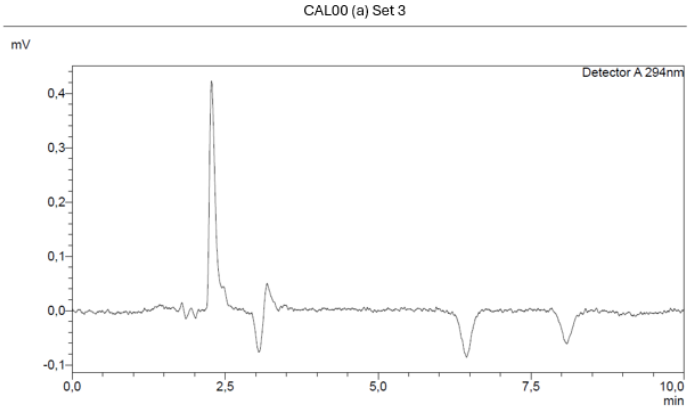

Figure 18. Chromatogram of CAL00 of set 3.

retention times of procaine and metronidazole (Figure 18). The negative peaks were most likely caused by pollution of the mobile phase due to recycling. At a certain point, the concentration of procaine and metronidazole in the mobile phase became excessively high, which leads to an increase of the baseline and negative peaks on the chromatogram. Therefore, the recycling of mobile phase was not conducted during validation sets 4, 5 and 6.

Moreover, it was evaluated if carry-over occurred based on the chromatograms of the calibration lines of sets 4, 5 and 6. Validation set 3 was not included in the evaluation of carry-over, since a baseline increase had been identified in the chromatograms of this set. As previously mentioned, two separate calibration lines were measured in each validation set. After every Upper Limit of Quantification (ULOQ) sample, the CAL00 of the second calibration line was measured. If carry-over had occurred, it would have shown on the chromatograms of the second CAL00. The baselines of the second CAL00 samples of sets 4, 5 and 6 were completely clean (Figure 19). It was confirmed that no carry-over had occurred in validation sets 4, 5, and 6.

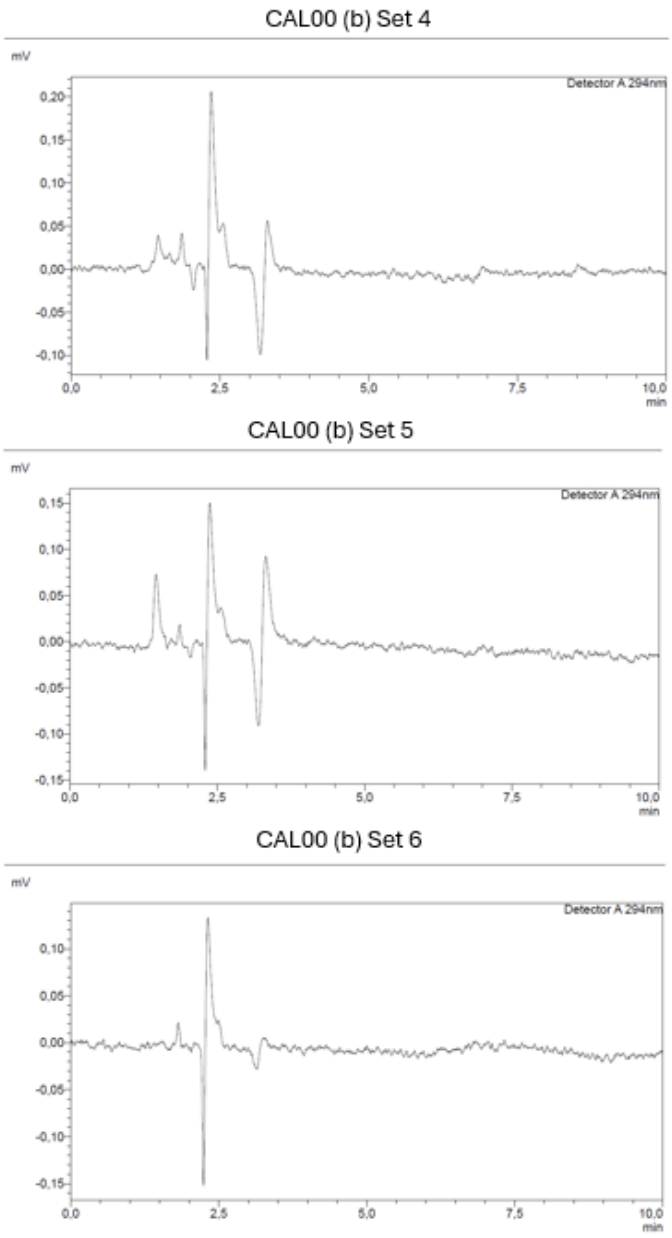

Figure 19. Chromatograms of the second CAL00 of sets 4, 5 and 6.

A normal linear regression was conducted for the calibration lines of every validation set using the ratios of the areas of procaine and metronidazole (y-axis) and the concentration of procaine hydrochloride in the calibration samples (x-axis). Then a back calculation was conducted and a weighing of 1/x was applied. This was decided, since a weighing of 1/x lead to the best results. The linearity was determined using data of 8 calibration lines (n=8). It should be noted that the data of set 3, which is included in the analysis, could be affected by the increased baseline. Nonetheless, it was decided to include the data of set 3, seeing that there was no acceptable reason to exclude the data. The linearity was calculated using the averages of the backcalculated concentrations of the calibration line samples and the averages of the nominal concentrations (= theoretical concentrations) of the calibration line samples of 8 calibration lines. The range of 0.5 to 500 µg/mL was proven to be linear (r^2 = 1.0000). Furthermore, the precision of the calibration line samples at every concentration level was calculated (Table 2), which was defined as the relative standard

deviation (RSD). The accuracy was defined as the deviation from the nominal concentration, which was calculated for the backcalculated calibration line samples (Table 3). The results showed that the precision (Table 2) and accuracy (Table 3) of the calibration line samples were all within limits. In conclusion, linearity was proven.

Table 2. Precision of the calibration line samples.

Backcalculated calibration line samples in µg procaineHCl per mL cardioplegic solution								
Nominal concentration (av)	0,576	1,152	5,76	11,52	57,6	115,2	576	
No	CAL0,5	CAL1,0	CAL5	CAL10	CAL50	CAL100	CAL500	r^2
31-1 calline 1	0,682	1,193	5,505	10,815	55,427	106,178	590,673	0,999600
31-1 calline 2	0,595	0,891	4,983	10,097	52,172	105,805	531,594	0,999997
10-2 calline 1	0,601	1,185	5,733	10,826	55,533	115,709	575,554	0,999984
10-2 calline 2	0,547	1,121	5,385	11,279	56,996	110,119	586,292	0,999853
12-2 calline 1	0,601	1,138	5,687	11,151	57,264	116,456	574,179	0,999990
12-2 calline 2	0,574	1,072	5,647	11,197	58,454	116,055	578,367	0,999853
17-2 calline 1	0,593	1,134	5,722	11,423	57,375	117,227	575,668	0,999985
17-2 calline 2	0,593	1,131	5,677	11,635	57,322	117,186	583,568	0,999996
Average (µg/mL)	0,598	1,108	5,542	11,053	56,318	113,092	574,487	
SD	0,038	0,095	0,256	0,475	1,951	4,936	18,289	
RSD (%) =reproducibility	6,41	8,62	4,61	4,30	3,46	4,36	3,18	
Limits	20%	15%	15%	15%	15%	15%	15%	

Table 3. Accuracy of the calibration line samples.

Backcalculated calibration line samples in % accuracy							
No	CAL0,5	CAL1,0	CAL5	CAL10	CAL50	CAL100	CAL500
31-1 calline 1	118,06	103,20	95,25	93,55	95,89	91,85	102,19
31-1 calline 2	104,23	78,02	87,27	88,41	91,37	92,65	93,10
10-2 calline 1	104,78	103,23	99,88	94,30	96,75	100,79	100,27
10-2 calline 2	95,35	97,63	93,81	98,25	99,30	95,92	102,14
12-2 calline 1	104,51	98,92	98,90	96,96	99,59	101,27	99,86
12-2 calline 2	99,75	93,25	98,21	97,37	101,66	100,92	100,59
17-2 calline 1	102,79	98,28	99,16	98,99	99,44	101,58	99,77
17-2 calline 2	102,85	97,97	98,39	100,82	99,35	101,55	101,14
Average (µg/mL)	104,04	96,31	96,36	96,08	97,92	98,32	99,88
SD	6,49	8,06	4,22	3,90	3,19	4,18	2,90
Limits	80-120%	85-115%	85-115%	85-115%	85-115%	85-115%	85-115%
	Significant outliers according to Grubbs test P<0,05 two sided						
	No outlier according to Grubbs, but deviates from limits						

The selection of concentrations for the QC samples was conducted based on ICH guidelines [5]. According to the ICH guidelines, the Lower Quality Control (LQC) is 3 times the LLOQ, the Middle Quality Control (MQC) is 30 to 50% of calibration line range and the High Quality Control (HQC) is 75% of the ULOQ [5]. The QC samples were at 500, 250, 25, 2.5 and 0.5 µg/mL procaine in matrix. The precision and accuracy of the QC samples within 4 validation sets were calculated (intraday) and of 4 validation sets combined (interday). The intraday precision was calculated by the standard deviation (SD) divided by the average concentration at every QC level. The intraday accuracy was calculated as the deviation from the nominal concentration at every QC level. The interday precision was calculated by combining the average backcalculated concentrations at every QC level of 4 validation sets and then calculating the RSD. The interday accuracy was calculated by combining the calculated accuracies at every QC level of 4 validation sets. The results showed that the LLOQ samples from set 3 had a greater deviation compared to the LLOQ samples of the other sets (Table 4). The reason behind this is the occurrence of an increased baseline in the measurements of set 3, due to the recycling of a small volume of mobile phase. In sets 4, 5 and 6 the mobile phase was not recycled during measurements and a fresh batch of mobile phase was used. In those sets there was no increased baseline. The accuracies of QC samples in the other three sets did not fall outside of limits (Tables 5, 6 and 7). In future measurements, mobile phase should be replaced regularly to avoid the issue of an increased baseline. Although the results of set 3 are not optimal due to issues with the baseline, the data was included in the analysis. It was concluded that the intraday accuracy and precision were all within limits. Furthermore, the interday accuracy and precision were all within limits (Table 8). All

of these findings support the reliability of the method. On top of that, it was proven that the method is still reliable after modifications are made to the method. In this case the modification was the recycling of mobile phase. The overall accuracy and precision still tested within limits, even with the less optimal results of set 3 included in the analysis. In conclusion, all of these findings support the robustness of the method.

Table 4. Accuracy and precision intraday of set 3.

Set 3						
sample	µg/mL	accuracy				limits
LLOQ 1	0,717	125,80	Av conc	0,684	µg/mL	
LLOQ 2	0,704	121,05	SD	0,024	µg/mL	
LLOQ 3	0,664	117,37	RSD	3,56	%	<20%
LLOQ 4	0,668	115,65	Av accuracy	119,11	%	80-120%
LLOQ 5	0,669	115,66	SD	4,34	%	
LQC 1	2,597	91,11	Av conc	2,66	µg/mL	
LQC 2	2,6	89,36	SD	0,059	µg/mL	
LQC 3	2,666	94,20	RSD	2,22	%	<15%
LQC 4	2,733	94,58	Av accuracy	92,47	%	85-115%
LQC 5	2,691	93,12	SD	2,20	%	
MQC 1	25,173	88,33	Av conc	27,35	µg/mL	
MQC 2	28,668	98,52	SD	1,82	µg/mL	
MQC 3	26,152	92,41	RSD	6,66	%	<15%
MQC 4	29,654	102,61	Av accuracy	95,12	%	85-115%
MQC 5	27,089	93,73	SD	5,55	%	
HQC 1	257,764	90,44	Av conc	265,8	µg/mL	
HQC 2	270,521	92,96	SD	4,97	µg/mL	
HQC 3	267,188	94,41	RSD	1,87	%	<15%
HQC 4	264,82	91,63	Av accuracy	92,49	%	85-115%
HQC 5	268,79	93,01	SD	1,51	%	
ULOQ 1	536,712	94,16	Av conc	531,7	µg/mL	
ULOQ 2	537,375	92,33	SD	8,57	µg/mL	
ULOQ 3	530,249	93,68	RSD	1,61	%	<15%
ULOQ 4	536,929	92,89	Av accuracy	92,51	%	85-115%
ULOQ 5	517,303	89,50	SD	1,83	%	
			No significant outliers according to Grubbs, but outside limits			

Table 5. Accuracy and precision intraday of set 4.

set 4						
sample	µg/mL	accuracy				limits
LLOQ 1	0,602	105,70	Av conc	0,586	µg/mL	
LLOQ 2	0,624	108,95	SD	0,026	µg/mL	
LLOQ 3	0,56	98,49	RSD	4,52	%	<20%
LLOQ 4	0,573	100,01	Av accuracy	102,42	%	80-120%
LLOQ 5	0,57	98,94	SD	4,66	%	
LQC 1	2,78	97,53	Av conc	2,72	µg/mL	
LQC 2	2,628	91,73	SD	0,068	µg/mL	
LQC 3	2,687	94,46	RSD	2,50	%	<15%
LQC 4	2,715	94,76	Av accuracy	95,09	%	85-115%
LQC 5	2,793	96,98	SD	2,31	%	
MQC 1	27,483	96,43	Av conc	27,80	µg/mL	
MQC 2	27,976	97,65	SD	0,21	µg/mL	
MQC 3	27,83	97,82	RSD	0,76	%	<15%
MQC 4	27,991	97,70	Av accuracy	97,17	%	85-115%
MQC 5	27,713	96,22	SD	0,77	%	
HQC 1	283,854	99,60	Av conc	285,0	µg/mL	
HQC 2	286,134	99,87	SD	2,48	µg/mL	
HQC 3	281,519	98,95	RSD	0,87	%	<15%
HQC 4	285,156	99,53	Av accuracy	99,60	%	85-115%
HQC 5	288,14	100,05	SD	0,42	%	
ULOQ 1	571,236	100,22	Av conc	570,4	µg/mL	
ULOQ 2	570,672	99,59	SD	3,45	µg/mL	
ULOQ 3	564,567	99,22	RSD	0,61	%	<15%
ULOQ 4	571,641	99,76	Av accuracy	99,68	%	85-115%
ULOQ 5	573,777	99,61	SD	0,36	%	

Table 6. Accuracy and precision intraday of set 5.

set 5						
sample	µg/mL	accuracy				limits
LLOQ 1	0,63	108,87	Av conc	0,585	µg/mL	
LLOQ 2	0,593	102,68	SD	0,044	µg/mL	
LLOQ 3	0,552	95,75	RSD	7,49	%	<20%
LLOQ 4	0,622	107,68	Av accuracy	101,36	%	80-120%
LLOQ 5	0,529	91,82	SD	7,43	%	
LQC 1	2,894	99,96	Av conc	2,82	µg/mL	
LQC 2	2,746	95,02	SD	0,078	µg/mL	
LQC 3	2,902	100,77	RSD	2,75	%	<15%
LQC 4	2,741	94,86	Av accuracy	97,62	%	85-115%
LQC 5	2,809	97,52	SD	2,73	%	
MQC 1	27,942	96,52	Av conc	28,48	µg/mL	
MQC 2	28,408	98,30	SD	0,53	µg/mL	
MQC 3	28,594	99,28	RSD	1,88	%	<15%
MQC 4	28,128	97,33	Av accuracy	98,65	%	85-115%
MQC 5	29,323	101,82	SD	2,05	%	
HQC 1	299,076	103,31	Av conc	293,9	µg/mL	
HQC 2	295,771	102,34	SD	3,54	µg/mL	
HQC 3	293,135	101,78	RSD	1,20	%	<15%
HQC 4	291,052	100,71	Av accuracy	101,81	%	85-115%
HQC 5	290,558	100,89	SD	1,07	%	
ULOQ 1	588,909	101,71	Av conc	584,7	µg/mL	
ULOQ 2	587,584	101,66	SD	3,45	µg/mL	
ULOQ 3	580,572	100,79	RSD	0,59	%	<15%
ULOQ 4	583,492	100,95	Av accuracy	101,26	%	85-115%
ULOQ 5	582,966	101,21	SD	0,41	%	

Table 7. Accuracy and precision intraday of set 6.

set 6						
sample	µg/mL	accuracy				limits
LLOQ 1	0,501	86,62	Av conc	0,556	µg/mL	
LLOQ 2	0,554	95,62	SD	0,041	µg/mL	
LLOQ 3	0,585	101,16	RSD	7,42	%	<20%
LLOQ 4	0,605	104,70	Av accurac	96,11	%	80-120%
LLOQ 5	0,533	92,45	SD	7,12	%	
LQC 1	2,77	95,84	Av conc	2,78	µg/mL	
LQC 2	2,885	99,66	SD	0,067	µg/mL	
LQC 3	2,729	94,43	RSD	2,41	%	<15%
LQC 4	2,78	96,18	Av accurac	96,07	%	85-115%
LQC 5	2,715	94,26	SD	2,18	%	
MQC 1	28,51	98,65	Av conc	28,75	µg/mL	
MQC 2	28,745	99,29	SD	0,36	µg/mL	
MQC 3	29,371	101,63	RSD	1,25	%	<15%
MQC 4	28,51	98,65	Av accurac	99,52	%	85-115%
MQC 5	28,619	99,37	SD	1,23	%	
HQC 1	292,769	101,30	Av conc	292,4	µg/mL	
HQC 2	292,245	100,95	SD	3,12	µg/mL	
HQC 3	297,397	102,91	RSD	1,07	%	<15%
HQC 4	290,042	100,36	Av accurac	101,21	%	85-115%
HQC 5	289,538	100,53	SD	1,02	%	
ULOQ 1	581,886	100,67	Av conc	580,5	µg/mL	
ULOQ 2	581,011	100,35	SD	3,01	µg/mL	
ULOQ 3	584,29	101,09	RSD	0,52	%	<15%
ULOQ 4	578,73	100,13	Av accurac	100,46	%	85-115%
ULOQ 5	576,438	100,08	SD	0,42	%	

Table 8. Accuracy and precision of all sets combined (interday) at every QC level. Sets 3 to 6 were included in the analysis.

	Precision					Accuracy			
	av conc			RSD (%)	Limits	Av accuracy			Limits
LLOQ	0,603	± 0,059	µg/mL	9,86 %	20%	LLOQ	104,75 ± 10,45	%	80-120%
LQC	2,74	± 0,088	µg/mL	3,21 %	15%	LQC	95,32 ± 2,90	%	85-115%
MQC	28,09	± 1,058	µg/mL	3,76 %	15%	MQC	97,61 ± 3,28	%	85-115%
HQC	284,3	± 11,95	µg/mL	4,20 %	15%	HQC	98,78 ± 3,94	%	85-115%
ULOQ	566,8	± 21,98	µg/mL	3,88 %	15%	ULOQ	98,48 ± 3,69	%	85-115%

The validation parameters that were not tested during this research project are stability and matrix effect. In this case stability is not as relevant, seeing that pharmaceutical products are used of which the stability during storage has already been confirmed. In order to test the matrix effect, patient material is needed which was not available during this research project. The stability and matrix effect could be tested in future research to complete the validation data for this method.

Although the linear ranges of other validated methods for procaine are smaller as opposed to the linear range of this method (0.5 to 500 µg/mL (r^2 = 1.0000)), it was necessary to test a larger range of concentrations [6-10]. Since the aim is to remove procaine from cardioplegic solution, large concentrations as well as very low concentrations of procaine would have to be measured. Therefore, the method had to be validated with a linear range that included the concentration of procaine in one IV bag of cardioplegic solution before and after filtration, 272 µg/mL and 2.72 µg/mL respectively. Furthermore, the LOD determined in the current method (0.073 µg/mL) was not as low as in other studies [6,7,8,10]. However, concentrations below 0.5 µg/mL did not have to be measured since the lowest concentration of interest was 2.72 µg/mL. A lower LOD was therefore not required to be determined. Another added value of this method is the matrix used in the samples. Matrices that are commonly used for the validation of procaine are equine plasma and urine, and human plasma [6-10]. To our knowledge, the validation of procaine in cardioplegic solution spiked with fetal bovine plasma was not yet conducted prior to this research project.

Solid Phase Extraction (SPE)

The adsorption capacity of activated carbon for procaine in pharmaceutical solution was determined in order to support the proof of principle of SPE. A solution of procaine hydrochloride in demineralized water was measured after adding no activated carbon and after adding 100 mg of activated carbon. The UV-spectra showed a clear decrease in absorption of the sample in which 100 mg activated carbon was added (Figure 20). The absorption decreased from 0.782 to 0.206. Based on these results, it was calculated that 74% of procaine was removed from the solution by adding 100 mg activated carbon. One IV bag of cardioplegic solution contains 272 mg in 1 Liter. An amount of 100 mg of activated carbon was able to remove 18.5 mg of procaine. This would mean that 1.5 grams of activated carbon is required to filter one IV bag of cardioplegic solution.

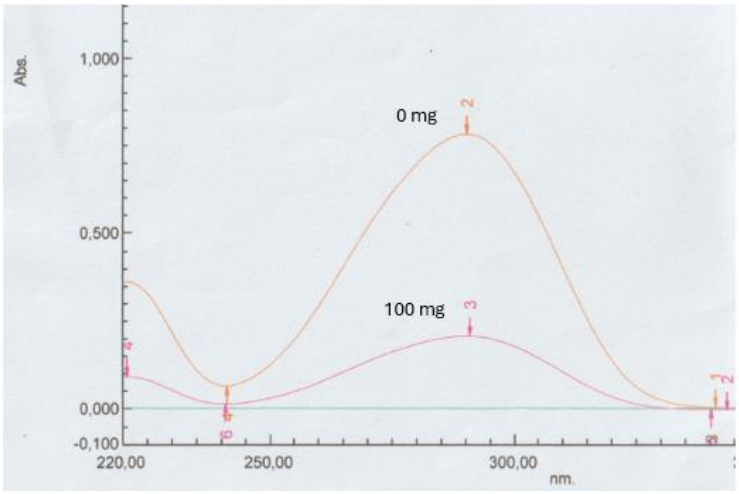

Figure 20. UV-spectra of a procaine solution in demineralized water (25 mg/50 mL) after adding no activated carbon (orange) and after adding 100 mg of activated carbon (pink). Both samples were 50 times diluted for measurement with UV/VIS-spectrometry.

Furthermore, the adsorption process over time was tested in order to determine the amount of time it takes for activated carbon to adsorb to procaine in the solution. It was determined that most of the adsorption occurs within 2 to 4 minutes after adding activated carbon to the solution (Figure 21). After 14 minutes, the decrease in absorption was minimal. The adsorption of activated carbon to procaine occurs in a short amount of time, which is an added benefit to this method.

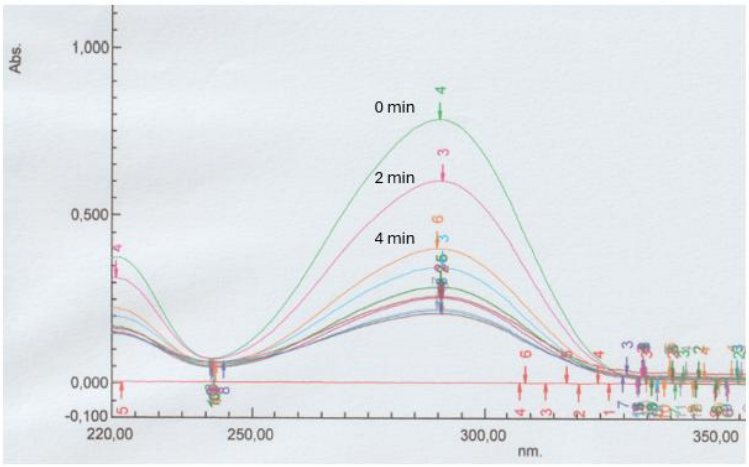

Figure 21. UV-spectra of a procaine solution in demineralized water (25 mg/50 mL) after adding 300 mg of activated carbon. A sample was taken every 2 minutes for up to 20 minutes. The most decrease in absorption is seen after 2 to 4 minutes.

The results of the activated carbon experiments showed that the method of using activated carbon to remove procaine from a solution is effective as well as time efficient. The main disadvantage of this method is the fact that a large amount of activated carbon will be required to filter several Liters of cardioplegic solution.

Conclusion

In this research project, a low-budget sustainable method for the filtration and quantification of procaine in used cardioplegic solution was developed. The HPLC method developed for the quantification of procaine was able to meet the tested validation criteria, including linearity, accuracy and precision, and selectivity. The LOD was determined to be at 0.073 µg/mL and the LLOQ was at 0.5 µg/mL. Additional validation criteria have yet to be determined for the current method. Moreover, the efficacy of filtration of procaine using activated carbon as a low-budget SPE material was determined. It was shown that 100 mg of activated carbon was required to filter 74% of procaine from a pharmaceutical solution. The filtration of one IV bag would therefore require an estimated 1.5 grams of activated carbon. Furthermore, an effort was made to incorporate the aspect of sustainability during this project. Unfortunately, the recycling of 300 mL mobile phase lead to an increased baseline due to excessive pollution, which mainly caused issues with the data of the LLOQ. It was concluded that the recycling of mobile phase can be applied during validation, provided that the volume of mobile phase is sufficiently large and is replaced regularly.

Although steps were taken to incorporate the aspect of sustainability, there is room for improvement. The recycling of larger volumes of mobile phase should be tested in future research, to determine the minimum volume required for mobile phase recycling during validation. The reduction of waste produced by the use of mobile phase would be a great addition to sustainable practices in the lab. Another main cause of waste was the use of plastic disposables, such as polypropylene microtubes. A large amount of microtubes were disposed of after single use during the validation process. The overall amount of waste could be minimized by replacing disposable microtubes by ones that can be reused. Furthermore, the large amount of HPLC vials that were disposed of also contributed to excessive waste. The vials that were used were equipped with inserts, since low volume samples were prepared. In theory, the vials could be reused after disposing of the inserts containing the sample. Prior to the possible implementation of reusing vials during validation, it should be tested to see if this affects the reliability of the acquired data.

Lastly, there is a plethora of additional experiments that could be carried out to expand upon the current method in future research. As previously mentioned, the initial aim was to develop a cartridge to filter one IV bag of cardioplegic solution. Although the amount of activated carbon required to filter one IV bag was estimated based on SPE experiments, an actual cartridge has yet to be developed. The cartridge would have to be tested on cardioplegic solution to determine the efficacy in removing procaine from the solution. This would have to be conducted by measuring the procaine concentration before and after application of the cartridge, along with the concentration of procaine inside of the cartridge. Moreover, the manner in which the cartridge could be attached to the IV bag has yet to be explored. An alternative way of using activated carbon to filter procaine from cardioplegic solution could be by mixing it together in bulk instead of using a cartridge. Additional alternative ways of carrying out the filtration with activated carbon could be further explored. On top of that, other types of activated carbon could be tested to determine the most cost-effective type of activated carbon to use as SPE material.

One of the future aims would be to expand upon the current method by extrapolating it to other compounds and other kinds of perfusates used in the hospital. For instance, the method could also be validated for heparin in one of the variations of cardioplegic solution. Another future aim would be to explore more possibilities regarding sustainable practices during the validation process, such as recycling of mobile phase. Ultimately, this low-budget sustainable method could be used as a stepping stone towards the development of a universal method for the filtration of APIs from perfusates.

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Appendix A. SOP-CAL-4

This document lists the SOP for validation work and was used from 29-01-2025 to 18-02-2025

Start of the day

- Use fresh water
- Transfer the required solutions from the freezer into a beaker containing warm water
- Prepare the required solutions:
    - Procaine S1
    - IS (metronidazole)
    - Blank matrix (10 mL blank perfusate concentrate + 2.5 mL fetal bovine serum + add to 50 mL demiwater)
- Prepare all polypropylene microtubes, etc.
- Run LC on 30% MeOH for 30 minutes, 100% MeOH for 30 minutes, 30% MeOH for 30 minutes, and then stabilize the LC on 1 mL/min mobile phase for at least 20 minutes

End of the day

- Dispose of liquid waste
- Dispose of solid waste and disposables

RP-HPLC Method 3
DGU-14A Degasser Shimadzu
FCV-10AL VP Pump Shimadzu
LC-10AD VP Liquid Chromatograph Shimadzu
SIL-10AD VP Auto Injector Shimadzu
SPD-10A VP UV-VIS Detector Shimadzu
CTO-10A VP Column Oven Shimadzu
SCL-10A VP System Controller Shimadzu

Column: XSelect-3
XSELECT CSH C18 5 µm
4.6x150mm Column
Part No: 186005290
Lot No: 0102310341
Waters

Mobile phase: 10% v/v MeOH + 10 mM Na-Citrate buffer pH 3
Flow: 1 mL/min
Injection volume: 5 µL
Wavelength: 294 nm

Blank matrix

Serum to use will be HyClone® Fetal bovine serum, triple 0.1µm sterile filtered research grade EU approved with Cat no: CH30160.03. No data available anymore on this product (not in production any more), but equivalent products have an average protein concentration of 35-45mg protein per Liter. For perfusate we estimate a protein concentration of 2g/L of 2 mg/mL (10 L of perfusate going through a total blood volume of 200 mL). Blank matrix will be prepared by adding 10 mL blank concentrated perfusate to 2.5 mL fetal bovine serum and demi water is added to make 50 mL of blank matrix.

Preparation of IS

For the preparation of internal standard, a solution of 100 mg metronidazole is dissolved to 25.0 mL with acetonitrile. For sample preparation, 50 µL of this IS solution will be added to 450 µL of matrix.

Preparation of stocks calibration line

Stock 1 (=S1) = 5 mg/mL in water: 50 mg in 10 mL

Stocks	[P] (mg/mL)	Amount P in 50 µL	Stock	Water (µL)
S1	5	250 µg	Weigh 57.7 mg procaine HCl in 10 mL of demiwater	
S2	1	50 µg	200 µL S1	800
S3	0.5	25 µg	100 µL S1	900
S4	0.1	5 µg	200 µL S3	800
S5	0.05	2.5 µg	100 µL S3	900
S6	0.01	0.5 µg	200 µL S5	800
S7	0.005	250 ng	100 µL S5	900

Preparation of calibration line samples

Standard	[P] in matrix (µg/mL)	Amount P in 500 µL matrix	Matrix (µL)	IS (µL)	Stock (50 µL)	Label
CAL500	500	250 µg	450	50	S1	500
CAL100	100	50 µg	450	50	S2	100
CAL50	50	25 µg	450	50	S3	50
CAL10	10	5 µg	450	50	S4	10
CAL5	5	2.5 µg	450	50	S5	5
CAL1	1	0.5 µ	450	50	S6	1
CAL0.5	0.5	0.25 µg	450	50	S7	0.5
CAL0	0	0	450	50	WATER	0
CAL00	0	0	450	ACN	WATER	00

Dilution of samples

- Put 450 µL matrix in 1.5 mL polypropylene microtube
- Add 50 µL of IS solution (or acetonitrile for double blank)
- Add 50 µL procaine stock (in different concentrations in water) from low to high concentration
- Vortex for 10 seconds
- Check liquid levels of the samples
- Add 700 µL of ice cold acetonitrile (-20 °C) and vortex immediately
- Centrifuge at max for 10 min at 7 °C
- Transfer 250 µL to a new polypropylene microtube
- Add 750 µL water
- Vortex sample for 10 seconds
- Centrifuge sample at max for 10 min at 7 °C
- Transfer 200 µL to a vial containing an insert
- Check for and remove air bubbles
- Label the vial and inject in LC system

Appendix B. SOP-QC-2

This document lists the SOP for validation work and was used from 29-01-2025 to 18-02-2025

Start of the day

- Use fresh water
- Transfer the required solutions from the freezer into a beaker containing warm water
- Prepare the required solutions:
    - Procaine QCS1
    - IS (metronidazole)
    - Blank matrix (10 mL blank perfusate concentrate + 2.5 mL fetal bovine serum + add to 50 mL demiwater)
- Prepare all polypropylene microtubes, etc.
- Run LC on 30% MeOH for 30 minutes, 100% MeOH for 30 minutes, 30% MeOH for 30 minutes, and then stabilize the LC on 1 mL/min mobile phase for at least 20 minutes

End of the day

- Dispose of liquid waste
- Dispose of solid waste and disposables

RP-HPLC Method 3
DGU-14A Degasser Shimadzu
FCV-10AL VP Pump Shimadzu
LC-10AD VP Liquid Chromatograph Shimadzu
SIL-10AD VP Auto Injector Shimadzu
SPD-10A VP UV-VIS Detector Shimadzu
CTO-10A VP Column Oven Shimadzu
SCL-10A VP System Controller Shimadzu

Column: XSelect-3
XSELECT CSH C18 5 µm
4.6x150mm Column
Part No: 186005290
Lot No: 0102310341
Waters

Mobile phase: 10% v/v MeOH + 10 mM Na-Citrate buffer pH 3
Flow: 1 mL/min
Injection volume: 5 µL
Wavelength: 294 nm

Blank matrix

Serum to use will be HyClone® Fetal bovine serum, triple 0.1µm sterile filtered research grade EU approved with Cat no: CH30160.03. No data available anymore on this product (not in production any more), but equivalent products have an average protein concentration of 35-45mg protein per Liter. For perfusate we estimate a protein concentration of 2g/L of 2 mg/mL (10 L of perfusate going through a total blood volume of 200 mL). Blank matrix will be prepared by adding 10 mL blank concentrated perfusate to 2.5 mL fetal bovine serum and demi water is added to make 50 mL of blank matrix.

Preparation of IS

For the preparation of internal standard, a solution of 100 mg metronidazole is dissolved to 25.0 mL with acetonitrile. For sample preparation, 50 µL of this IS solution will be added to 450 µL of matrix. The internal standard solution that was prepared and used for the calibration line will be used for the preparation of the QC samples.

Preparation of stocks QC samples

QC Stock 1 (=QCS1) = 5 mg/mL in water: 50 mg in 10 mL

Stocks	[P] (mg/mL)	Amount P in 50 µL	Stock	Water (µL)
QCS1	5	250 µg	Weigh 57.7 mg procaine HCl in 10 mL of demiwater	
QCS2	2.5	125 µg	500 µL QCS1	500
QCS3	0.25	12.5 µg	100 µL QCS2	900
QCS4	0.025	1.25 µg	100 µL QCS3	900
QCS5	0.005	0.25 µg	200 µL QCS4	800

Preparation of QC samples

QC	[P] in matrix (µg/mL)	Amount P in 500 µL matrix	Matrix (µL)	IS (µL)	Stock (50 µL)	Label
ULOQ	500	250 µg	450	50	QCS1	UL
HQC	250	125 µg	450	50	QCS2	H
MQC	25	12.5 µg	450	50	QCS3	M
LQC	2.5	1.25 µg	450	50	QCS4	L
LLOQ	0.5	0.25 µg	450	50	QCS5	LL

Dilution of QC samples

- Put 450 µL matrix in 1.5 mL polypropylene microtube
- Add 50 µL of IS solution
- Add 50 µL procaine stock (in different concentrations in water) from low to high concentration
- Vortex for 10 seconds
- Check liquid levels of the samples
- Add 700 µL of ice cold acetonitrile (-20 °C) and vortex immediately
- Centrifuge at max for 10 min at 7 °C
- Transfer 250 µL to a new polypropylene microtube
- Add 750 µL water
- Vortex sample for 10 seconds
- Centrifuge sample at max for 10 min at 7 °C
- Transfer 200 µL to a vial containing an insert
- Check for and remove air bubbles
- Label the vial and inject in LC system