universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

„Stability of native and modified UDP-sugars“

verfasst von

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Acknowledgments

Firstly, I would like to thank my supervisor Prof. Dr. Mag. Pharm Ernst Urban for his excellent supervision.

I would also like to thank Dr. Gerd Wagner for giving me the great opportunity to join his team and work on this exciting project. I am deeply grateful of his help in the competition of this diploma thesis.

I am also greatly indebted to all the members of the BioChemistry Lab for their great help and advice to me.

Finally, I would express my deep gratitude to my parents for their support and encouragement.

Zusammenfassung

Die wissenschaftlichen Experimente für die vorliegende Diplomarbeit wurden im „King's College London, School of Biomedical Sciences Institute of Pharmaceutical Science & Department of Chemistry" im Zeitraum von Anfang März bis Ende Juni 2013 unter der Leitung von Herrn Dr. Gerd Wagner durchgeführt.

In den letzten Jahren wurde immer mehr über die wichtige Rolle der Glykosylierung bei der Regulierung der Biologischen Aktivität berichtet.

Die Biologische Glykosylierung wird hauptsächlich von Glykosyltransferasen ausgetragen. Diese Enzyme katalysieren den Transfer von Mono- oder Oligosacchariden vom Glycosyldonor zum Akzeptormolekül wie Lipide, Saccharide und Proteine. Die Glykosyltransferasen nutzen aktivierte Formen von Zucker Nukleotiden wie zum Beispiel UDP-Galactose als Donoren für die Glykosylierungs Reaktion. Die Inhibition der Glykosyltransferasen mittels Zucker Nukleotid Analogen stellt daher eine vielversprechende Strategie zur Entwicklung neuer Wirkstoffe zur Behandlung unterschiedlichster Krankheiten. Ausgehend von den Erfolgen der Forschungsgruppe von Dr. Wager, die UDP-Zucker Analoga wie 5-(5-formylthien-2-yl) UDP-Gal als eine neue Klasse von allosterische Inhibitoren entwickelt haben, untersuchte ich im Rahmen meiner Arbeit die Stabilität von Nativen UDP-Zuckern, sowie dessen modifizierten Analoga.

Die UDP-Zucker die zu den Stabilitätstest herangezogen wurden waren UDP-Gal, UDP-GlcNAc und 5-(5-formylthien-2-yl) UDP-Gal.

Die Medien die für die Stabiliätstests verwendet wurden waren Wasser, NaCl-Lösung und biologisches Medium (RPMI 1640 medium, Gluta MAX, 2%FBS and 100 units of penicillin and 100µg of streptomycin per ml)

Die Auftrennung und Identifizierung der Zucker-Nukleotid Spaltprodukte erfolgte mittels HPLC mit UV/Vis Detektor.

Table of Contents

1. Introduction

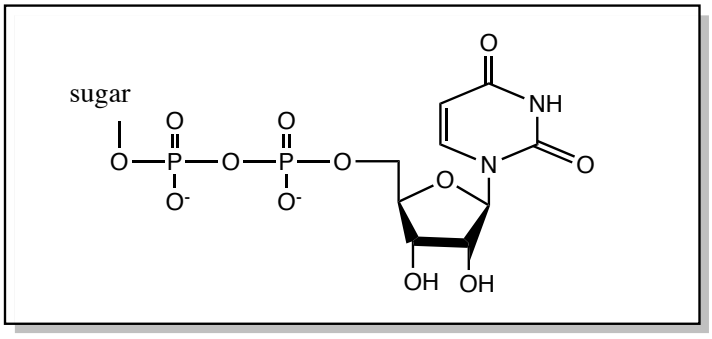
1.1 General background

Glycosylation plays an important role in regulating biological activities, including cellular adhesion and cell signalling.[1-4] The Biological glycosylation is mainly carried out by glycosyltransferases. These enzymes catalyse the transfer of a mono- or oligo-saccharide from a glycosyl donor to an acceptor-molecule.[5-8] The glycosyltransferases are a large enzyme family with various sequences and functions, but with a noticeably conserved three-dimensional structure.[9-11] In eukaryotic cells glycosylation is carried out in the endoplasmatic reticulum and/or Golgi apparatus by glycosyltransferases which are predominantly type-2 trans- membrane proteins.[12]

Glycosyltransferase acceptors belong to a wide range of structural classes, for example proteins, saccharides, lipids and secondary metabolites.[5-8] Glycosyltransferases use activated forms of sugar nucleotides,[13] such as UDP-galactose, or lipid-linked sugars such as dolicholphosphate mannose,[14] as donors for the glycosylation reaction.

So far, nine different sugar-nucleotide donors have been found in mammalian cells.[15]

In this diploma thesis, we will focus on UDP-sugars, see Figure 1, which are donors for the glycosyltransferases, these play an essential role in the biosynthesis of carbohydrates and glycoconjugates, which are involved in various diseases and metabolic disorders[16].



Fiugre 1. Structure of a uridine diphosphate sugar

For example, UDP-Gal is the major donor in the biosynthesis of Sialyl Lewis X[17]. The tetrasaccharide Sialyl Lewis X is involved in cell adhesion and therefore it plays an important role in inflammation and cancer metastasis. Consequently, inhibition of glycosyltransferases with sugar-nucleotide analogues is a promising strategy for the discovery of novel drugs for treating different diseases[18].

Since current research in multiple disease areas focuses on glycosyltransferase inhibitors, a multitude of functional assays have been developed.

Ongoing efforts in Gerd Wagner research group utilise UDP-sugars as novel chemical probes and enzyme inhibitors such as 5-(5-formylthien-2-yl) UDP-Gal, see Figure 2, as a new class of allosteric galactosyltransferase inhibitors[18, 23].

The development of robust assays requires sufficient stability of all components under the respective assay conditions. However, few studies have been published on the stability of UDP-glycosides so far.

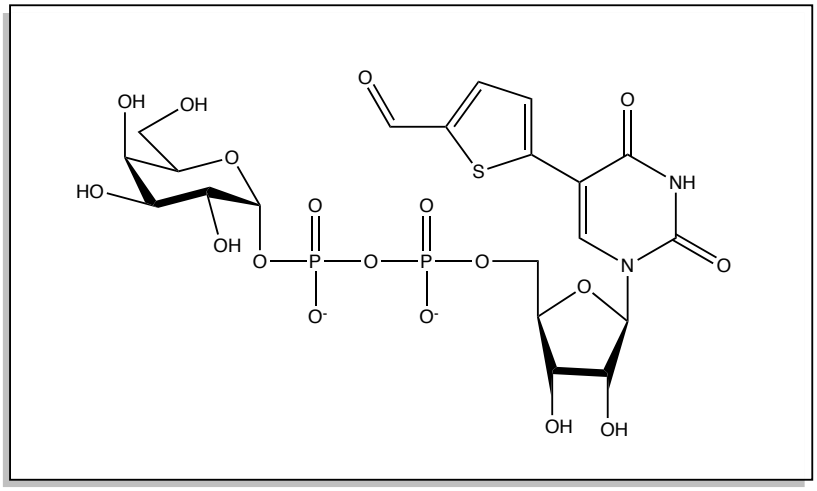


Figure 2. Structure of 5-(5-formylthien-2-yl) UDP-Gal

1.2 Stability of UDP-sugars

There are different potential sites for the hydrolysis of the UDP-glycosides, see Figure 3. The molecule can hydrolyse at the pyrophosphate bond, at the at the two phosphoester bonds or at the bond of uracil and ribose. In this diploma thesis, the stability of UDP-sugars will be discussed, including the major breakdown products of the UDP-sugars.

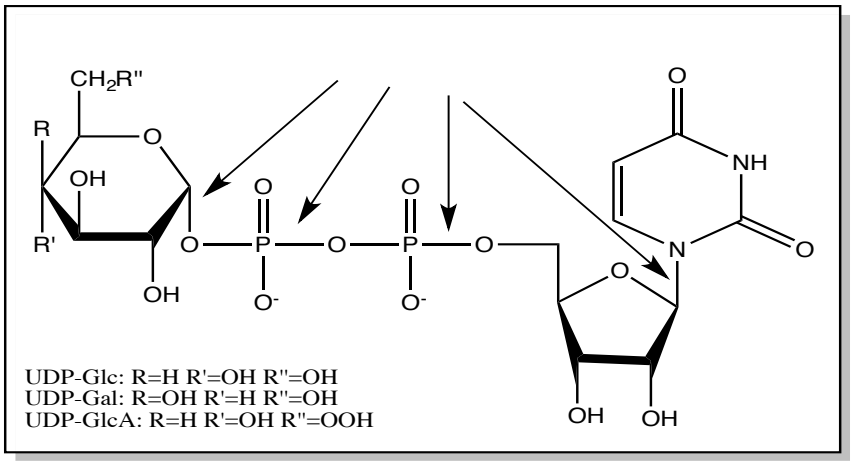


Figure 3. Potential sites of hydrolysis

1.2.1 pH dependent decomposition of UDP-sugars

Huhta et al.[19] studied the chemical hydrolysis of nucleoside diphosphate sugars and found the reaction to be pH dependent. The rate of UDP-Glc hydrolysis was slowest around pH 6.0, and increased markedly at alkaline or acidic pH values, see Figure 4.

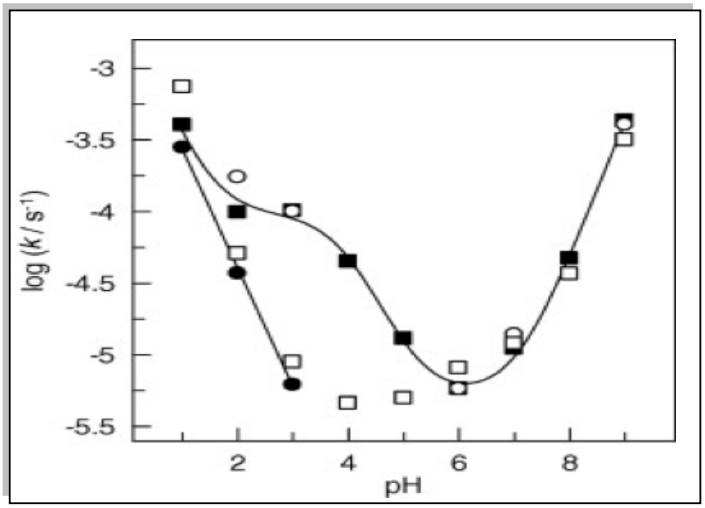
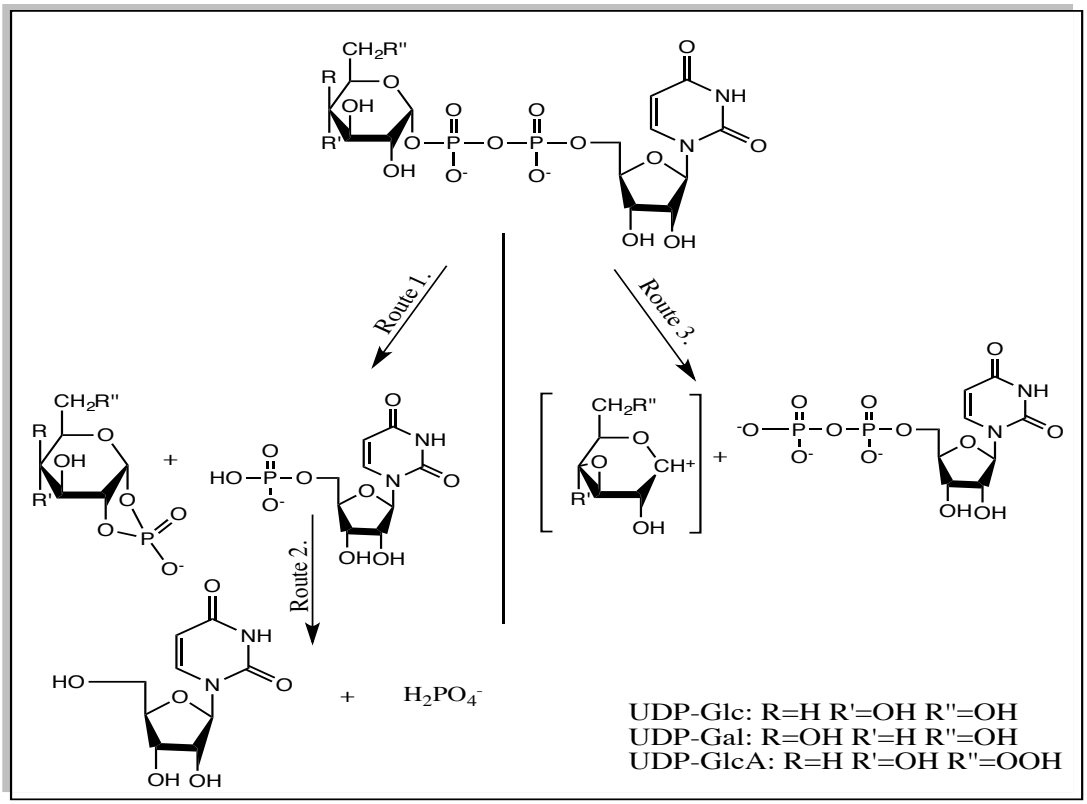


Figure 4. pH rate profiles of total disappearance of UDP-Glc filled squares, at 90 °C and ionic strength of 0.1 M. (Huhta et al. 2009)

The group detected UMP as the predominant decomposition product of the UDP-Glc hydrolysis across the pH range studied, see Scheme 1, Route 1. They also detected that UMP dephosphorylates between pH 5-7, see Scheme 1, Route 2.

Huhta et al.[19] also mention that the decomposition reaction of UDP-Glc is an S_N2-type reaction. The hydroxyl group at the 2 position of the pyranose sugar attacks the phosphate, the products of this reaction are 1,2-cyclic sugar phosphate and 5'-UMP.

In contrast to Huhta et al., Bedford[21] reported the primary hydrolysis product of UDP-Glc at pH 1-3 to be UDP, see Scheme 1, Route 3. In the work of E. Huhta[19], no uridine diphosphate was found and it was suggested that the decomposition from UDP-Glc to UDP can occur under conditions where a, stabilised carbocation intermediate is formed, or a nucleophile is available at close proximity.



Scheme 1. Reaction routes suggested for the decomposition of UDP-sugars

1.2.2 The metal dependent decomposition of UDP-sugars

Sugiura et al.[20] studied the effects of Mn^{2+} ions on the stability of UDP-Glc, UDP-Gal, UDP-GlcA, UDP-GlcNAc and UDP-GalNAc. They suggested, that the equatorial 2-OH group on the pyranose sugar is important for the reaction. UDP-GlcA, UDP-Glc, and UDP-Gal, decomposed in presence of $Mn^{2+,}$ while the sugars with an N-acetyl group on the second position on the pyranose ring, such as UDP-GlcNAc and UDP-GalNAc remaind in tact under the same conditions.

Sugiura et al.[20] showed the effect of UDP-GlcA and UDP-Gal-NAc , in the presence of Mn2+ ions, under different Mn^{2+} concentrations, temperatures and pH values.

UDP-GlcA was stable in the absence of $MnCl_2$, whilst the amount of decomposition of UDP-GlcA increased to 100% after 18 hours incubation in 20 mL $MnCl_2$. UDP-GalNAc was stable after 18 hours. The decomposition rate of UDP-GlcA rises with a increasing concentration of $MnCl_2$. The decomposition rate also rises with temperature in the presence of $MnCl_2$. The decomposition rate stays stable at around pH 6.0 and rises at high values. Therefor the results of Sugiura et al. showed that the stability of UDP-GlcA is depended on the Mn^{2+} concentration, the temperature and the pH value.

The Nunez et al.[22] demonstrated the strong effect of Mn^{2+} on the decomposition of UDP-Glc and UDP-Gal in different pH conditions. The decomposition rate of both UDP-sugars increased with an increasing concentration of Mn^{2+}, and with higher pH values. They showed that a different conformation affected the decomposition rate significantly.
UDP-Gal decomposes much faster than UDP-Glc under all conditions tested. The axial 4-OH group of the pyranose from UDP-Gal may effect the faster decomposition in comparison to UDP-Glc.[22]

The results of the studies showed that in absence of metal ions UDP sugars are stable at neutral pH values[22,19] even under elevated temperatures[19]. Acidic and basic conditions promote the decomposition reaction of UDP sugars with a hydroxyl group at the 2 position of the pyranose sugar[19]. Some metal ions, such as Mn^{2+} catalyse the decomposition of UDP-Glc,[22] UDP-Gal,[22] UDP-GlcA[20] even at neutral pH values.
UDP-sugars with a 2-OH group in the equatorial position of the pyranose sugar such as UDP-GlcA[20], UDP-Gal[22], and UDP-Glc[22] undergo a nucleophilic attack reaction.
The hydroxyl group at the 2 position of the pyranose sugar attacks the α diphosphate, the products of this reaction are 1,2-cyclic sugar phosphate and 5'-UMP. The sugars with a different substituent on the 2 position of the pyranose sugar such as UDP-GalNAc[20], and UDP-GlcNAc[20] are stable under the same conditions. The axial 4-OH group of the pyranose from UDP-Gal may effect the faster decomposition in comparison to UDP-Glc.[22]

2. Analysing the stability of UDP-Gal

2.1 Methodology development

Finding a suitable method to separate the nucleotides.

According to the literature reverse-phase HLPC, utilizing UV detection, is the method of choice, for analysing the stability of UDP-sugars and quantify the cleavage products. The main disadvantage of this method is the long retention time for the UDP-sugars. Due to this disadvantage, different reverse-phase HPLC columns were compared with an ion exchange HPLC column.

2.1.1 Investigation of retention times of uridine diphosphate galactose, uridine diphosphate and uridine monophosphate by ion exchange HPLC.

Each of the nucleotide samples was dissolved in ultra pure H_2O (0.5 in 200 µL) and tested separate. Method details are shown in Table 1.

Apparatus	Aglinet 1100 Seris DAD detector		
Column	SOURCE 15Q, 6mm x 1,5 cm Hl. Glas colum		
Solvent A	Water		
Solvent B	2M NaCl		
Detection wavelength (nm)	254.8		
Reverence wavelength (nm)	Ref=700,40		
Flow (ml/min)	2ml/min		
Pump Program	Method 1		
Inj. Volum	30 µL	3 µL	2 µL
	Time	A%	B%
	0	100	0
	1.5	100	0
	4.5	70	30
	5.0	50	50
	6.0	50	50
	7.0	100	0
	9	100	0
Retention Times graphs are not shown	UMP -3.569 min	UDP-3.982 min	UDP-Gal -3.614min

Table 1.HPLC conditions used for investigation of retention times of UMP, UDP and UDP-Gal with ion exchange colum

After the retention times of the single nucleotides were investigated, it was noticed that UDP-Gal and UMP have similar retention times. In order to find a suitable method to separate the nucleotides in the mixture, different methods were used. Each of the nucleotide samples were dissolved in ultra pure H_2O, 1mM and tested in a 1:1:1 mixture (333 µM of each nucleotide in the mixture). Method details are shown in Table 2.

Apparatus	Aglinet 1100 Seris DAD detector								
Column	SOURCE 15Q, 6mm x 1,5 cm Hl. Glas colum.								
Solvent A	Water								
Solvent B	2 M NaCl								
Detection wavelength (nm)	254.8								
Reverence wavelength (nm)	Ref=700,40								
	Method 1			Method 2			Method 2		
Inj. Volum	2.0µL			2.0µL			5.0µL		
	Pump Program			Pump Program			Pump Program		
Flow (ml/min)	T	A%	B%	T	A%	B%	T	A%	B%
1	0	100%	0	0	100	0	0	100	0
1	1.5	0	0	1.5	100	0	1.5	100	0
2	1.6	95	5	1.6	100	0	1.6	100	0
2	5.5	80	20	60	85	14	60	93	7
2	6	50	50	61	50	50	61	50	50
2	7	50	50	62	50	64	62	50	50
2	8	100	0	64	100	0	64	100	0
2	10	100	0	66	100	0	66	0	0
Retention Times Graphs are not shown	Big peak at 3.407 min presumably from UDP-Gal and UMP; UDP 3,992 min			UMP 10.407 min UDP-Gal 10.784 min UDP 18.604 min The peaks of UMP and UDPgal were not well separated (overloaded)			UMP 16.332 min UDP-Gal 16.904 min UDP28.700 min The peaks of UMP and UDPgal were not well separated (overloaded)		

Table 2. HPLC conditions used for investigation of retention times of the mixture of UMP, UDP and UDPgal with ion exchange colum.

2.1.2 Investigation of retention times of uridine diphosphate galactose, uridine diphosphate and uridine monophosphate by reversed-phase HPLC.

2.1.2.1 Investigation of retention times with an Alltima C18, 3 µ, 150mm 4.6 mm column

Each of the nucleotide samples were dissolved in ultra pure H_2O, 1mM and tested separate. Method details are shown in Table 3.

Apparatus	Aglinet 1100 Seris DAD detector		
Column	Alltima C18, 3 µ, 150mm 4.6 mm		
Solvent A	100mM Potassium Phosphate + 8mM tetrabutylammoniumhydrogensulfat pH 6.5		
Solvent B	30% MeOH +70 Solvent A		
Detection wavelength (nm)	254.8		
Reverence wavelength (nm)	Ref=700,40		
Inj. Volum	10 µL		
Flow (ml/min)	1ml/min		
Pump Program	Method 1		
	Time	A%	B%
	0	100	0
	2	100	0
	17	50	50
	19	50	50
	20	100	0
	25	100	0
Retention Times Graphs are not shown	UDP-Gal - 7.482 min UDP - 9.631 min UMP - 7.074 min		

Table 3.HPLC conditions used for investigation of retention times of UMP, UDP and UDPgal with ion exchange colum

After the retention times of the single nucleotides were investigated, it was noticed that UDP-Gal and UMP have similar retention times. In order to find a suitable method to separate the nucleotides in the mixture, different methods were used. Each of the nucleotide samples were dissolved in ultra pure H_2O, 1mM and tested in a 1:1:1 mixture (333 µM of each nucleotide in the mixture). Method details are shown in Table 4.

Apparatus	Aglinet 1100 Seris DAD detector											
Column	Alltima C18, 3 µ, 150mm 4.6 mm											
Solvent A	100mM Potassium Phosphate + 8mM tetrabutylammoniumhydrogensulfat pH 6.5											
Solvent B	30% MeOH +70 Solvent A											
Detection wavelength (nm)	254.8											
Reverence wavelength (nm)	Ref=700,40											
	Method 1			Method 2			Method 3			Method 4		
Inj. Volum	10 µL			10 µL			10 µL			30 µL		
	Pump Program			Pump Program			Pump Program			Pump Program		
Flow (ml/min)	T	A%	B%	T	A%	B%	T	A%	B%	T	A%	B%
1	0	100	0	0	100	0	0	100	0	0	100	0
1	2	100	0	2	100	0	2	100	0	2	100	0
1	17	80	20	17	50	50	17	95	5	17	50	50
1	19	50	50	19	50	50	19	95	5	19	50	50
1	21	50	50	20	100	0	20	100	0	20	100	0
1	25	100	0	25	100	0	25	10	10	25	100	0
Retention Times Graphs are not shown	Two overlapping peaks at 7.805 min and 9.239 min			Two overlapping peaks at 7.893 min and 8.905 min			Two overlapping peaks at 8.211 min and 9.747 min			First peak at 8.601min presumably from UMP, second peak at 9.747 min presumably from UDP-Gal and third at 11,92 min presumably from UDP, the peaks were not perfectly separated		

Table 4. HPLC conditions used for investigation of retention times of the mixture of UMP, UDP and UDPgal with i Alltima C18, 3 µ, 150mm 4.6 mm colum

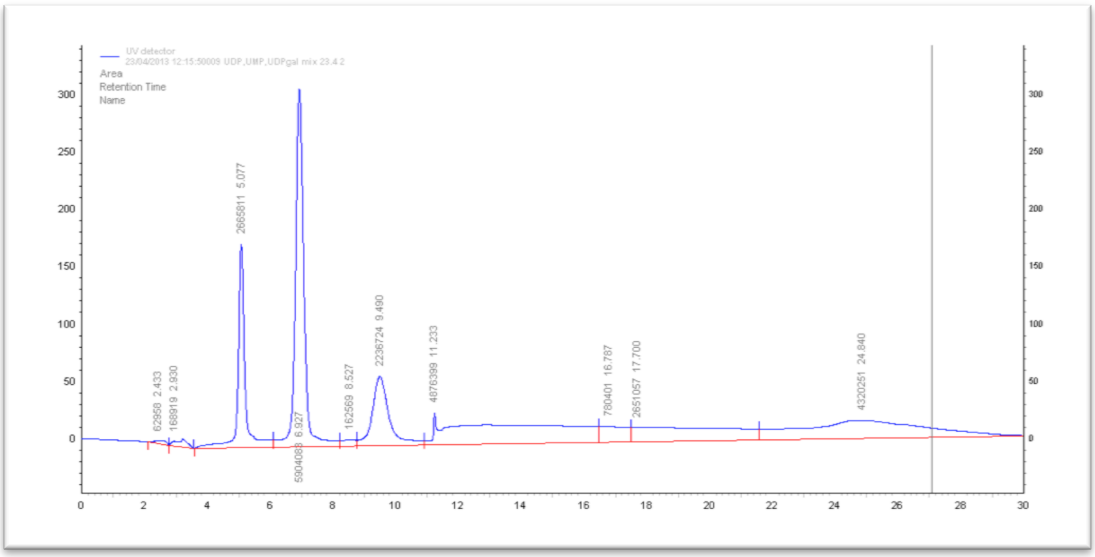
Multiple attempts to reproduce and improve the results with the Alltima C18, 3 µ, 150mm 4.6 mm Column were unsuccessful.

2.1.2.2 Investigation of retention times with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column

Each of the nucleotide samples were dissolved in ultra pure H_2O, 1mM and tested in a 1:1:1 mixture (333 µM of each nucleotide in the mixture). Method details are shown in Table 5. and Table 6. Separation peaks of the sample are shown in Figure 4. and Figure 5

	Method 1		
Apparatus	HPLC setup consisting of Jasco LC-Net II/ADC Interface, Jasco PU-2089 Plus Quaternary gradient pump, Jasco UV-2075 Plus Intelligent UV/Vis Detector and Jasco AS-2050 Plus Autosampler		
Column	Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column		
Solvent A	100mM Potassium Phosphate + 8mM tetrabutylammoniumhydrogensulfat pH 6.5		
Solvent B	30% MeOH +70% Solvent A		
Column Oven	Oven temperature 30.0 deg C		
Detection Wavelength (nm)	254		
Inj. Volum	30 µL		
	Pump Program		
Flow (ml/min)	T	A%	B%
1	0	100	0
1	2	100	0
1	17	80	20
1	21	50	50
1	22	50	50
1	30	100	0
Retention Times	First peak at 5.077 min from UMP, second peak at 6.927 min from UDP-Gal and third at 9.490 min from UDP. Details are shown in HPLC Graph 1.		

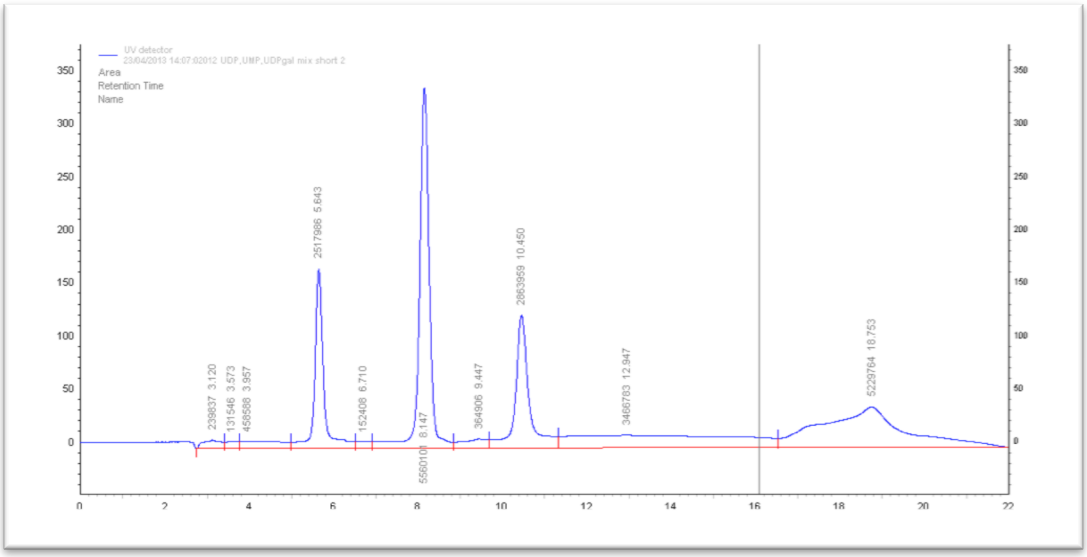
Table 5. Method 1. HPLC conditions used for investigation of retention times of the mixture of UMP, UDP and UDP-Gal with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column



HPLC Graph 1. Method 1. Time point profiles of UMP, UDP and UDP-Gal with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column

	Method 2		
Apparatus	HPLC setup consisting of Jasco LC-Net II/ADC Interface, Jasco PU-2089 Plus Quaternary gradient pump, Jasco UV-2075 Plus Intelligent UV/Vis Detector and Jasco AS-2050 Plus Autosampler		
Column	Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column		
Solvent A	100mM Potassium Phosphate + 8mM tetrabutylammoniumhydrogensulfet pH 6.5		
Solvent B	30% MeOH +70% Solvent A		
Column Oven	Oven temperature 30.0 deg C		
Detection Wavelength (nm)	254		
Inj. Volum	30 µL		
	Pump Program		
Flow (ml/min)	T	A%	B%
1	0	100	0
1	2	100	0
1	12	85	15
1	13	50	50
1	16	50	50
1	17	100	0
	22	100	0
Retention Times	First peak at 5.643 from UMP, second peak at 8.147 from UDPgal and third at 10.450 from UDP. Details are shown in HPLC Graph 2.		

Table 6. Method 2. HPLC conditions used for investigation of retention times of the mixture of UMP, UDP and UDP-Gal with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column



HPLC Graph 2. Time point profiles of UMP, UDP and UDP-Gal with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column

According to the investigation of retention times, the second method was reproducible had a better separation of the nucleotides in compare to the first method and the different HPLC columns tested. This method seemed suitable for the further experiments.

2.1.3 Creating calibration curves

In order to quantify UDPgal and its degradation products precisely calibration curves of UDPgal, UMP and UDP must be developed.

2.1.3.1 Calibration curve for UMP

Based on the developed method "Method 2" see 2.2.2.2

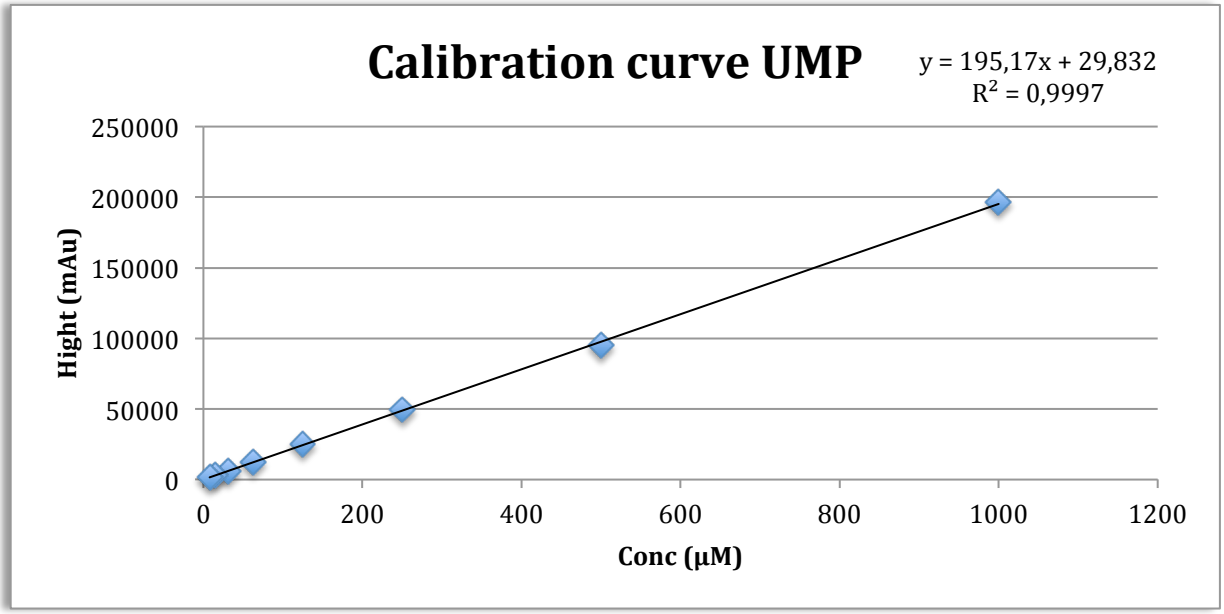


Figure 6. Calibration curve of UMP with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column

The height was chosen as the measurement of choice to create the calibration curves, because its measurements were better reproducible and the graph showed a better linearity in comparison to the area. Details are shown in Table and Figure

Conc (uM)	Height 1 (mAu)	Height 2 (mAu)	Average	Standard deviation	relative standard deviation
1000	197.859	194.699	196279	2234,457429	1,13840881
500	96.382	93.401	94891,5	2107,885315	2,221363678
250	49.644	49.652	49648	5,656854249	0,011393922
125	25.239	24.620	24929,5	437,6990976	1,755747598
62,5	12.337	12.374	12355,5	26,1629509	0,211751454
31,25	6.150	6.152	6151	1,414213562	0,022991604
15,625	3.148	3.086	3117	43,84062043	1,406500495
7,813	1.723	1.633	1678	63,63961031	3,792587027

Conc (uM)	Area 1	Area 2	Average	Standard deviation	relative standard deviation
1000	2.360.691	2.196.389	2278540	116179,0584	5,09883778
500	1.075.958	1.026.012	1050985	35317,15529	3,360386237
250	548.451	547.079	547765	970,1505038	0,177110714
125	301.754	279.291	290522,5	15883,73963	5,467301027
62,5	147.825	146.975	147400	601,040764	0,407761712
31,25	81.559	80.956	81257,5	426,3853891	0,52473358
15,625	62.413	49.551	55982	9094,80742	16,24594945
7,813	41.472	24.412	32942	12063,24169	36,61963963

Table Calibration data of UMP with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column

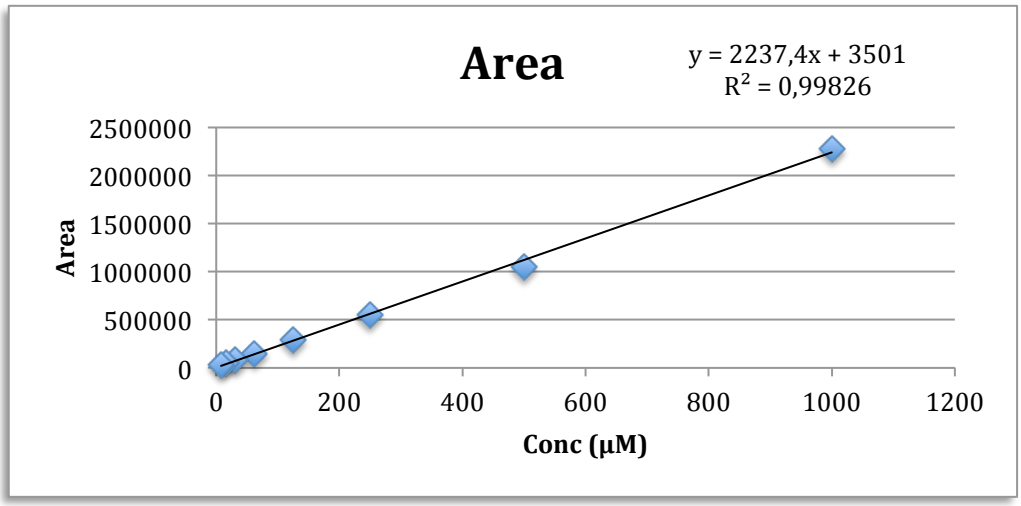


Figure Calibration data of UMP with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column

2.1.3.2 Calibration curve for UDP

Based on the developed method "Method 2" see 2.2.2.2

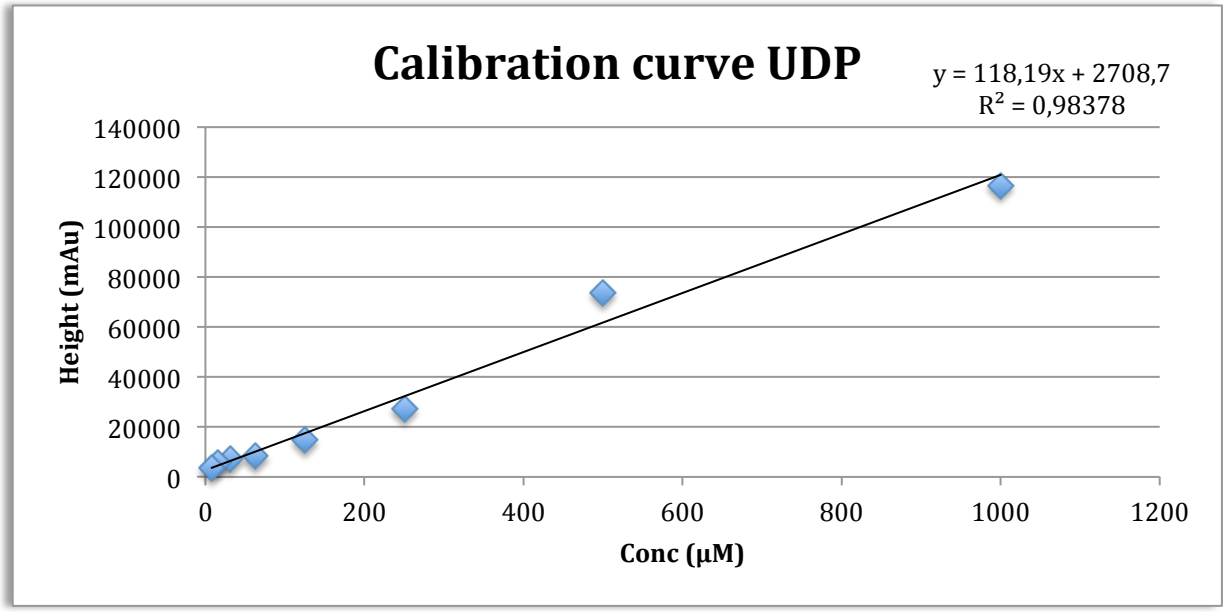


Figure 7. Calibration curve of UDP with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column

2.1.3.3 Calibration curve for UDPgal

Based on the developed method "Method 2" see 2.2.2.2

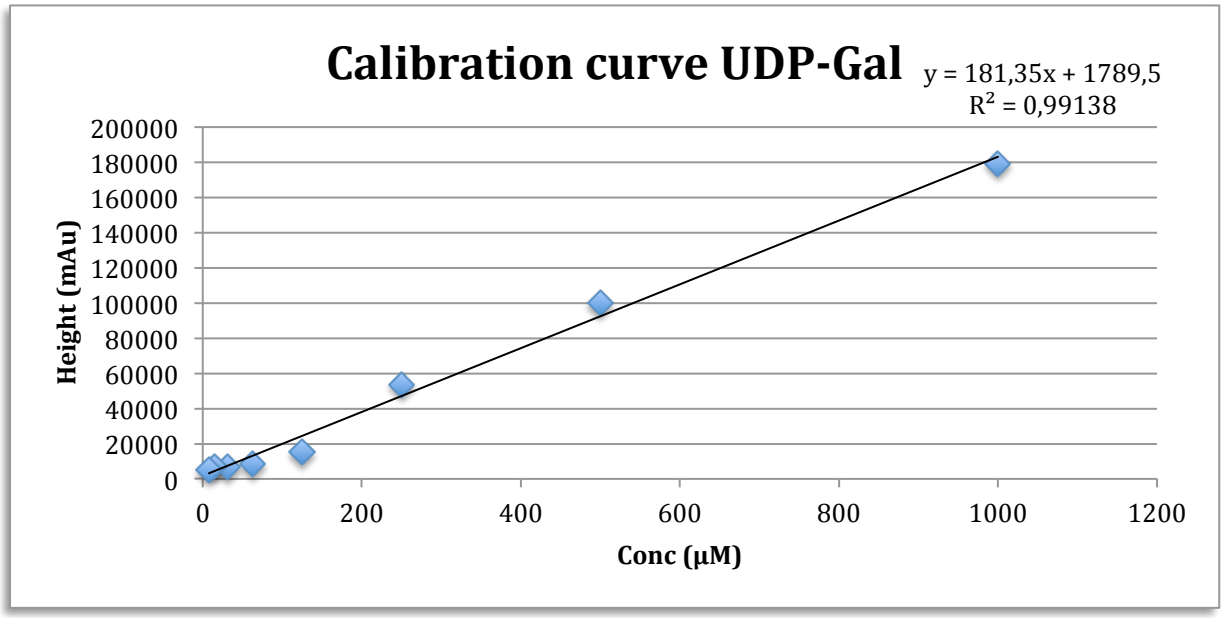


Figure 8. Calibration curve of UDPgal with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column

2.1.4 Finding a method to separate solids from biological media

Comparison of Centrifugation and Filtration Techniques to separate solids from biological media (RPMI 1640 medium, Gluta MAX, 2%FBS and 100 units of penicillin and 100µg of streptomycin per ml) which can block the HPLC column. Comparing 13mm Syringe Filters w/ 0.2 µm PTFE with centrifuge technic using a technico mini centrifuge. UMP was dissolved in biological media, 1mM.

2.1.4.1 Filtration technique

Each time 300µL of the 1mM UMP biological media were transferred into the syringe and filtered with the syringe filters. The samples got frozen with dry ice after filtration to stop the degradation of the Molecule. The samples got frozen with dry ice after filtration to stop the degradation of the Molecule.

Conc (uM)	Retention Time 1	Retention Time 2	Retention Time 3	Average	Standard deviation	relative standard deviation
1000	4,863	5,377	5,480	5,24	0,330528365	6,307793223
Conc (uM)	Height 1	Height 2	Height 3	Average	Standard deviation	relative standard deviation
1000	290.474	261.905	319447	290.609	28771,23637	9,900336663
Conc (uM)	Area 1	Area 2	Area 3	Average	Standard deviation	relative standard deviation
1000	3.445.945	3.274.211	4345273	3688476,333	575247,3709	15,59579943

Table 7. . HPLC results for the Filtration technic of 1mM UMP biological media with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column

2.1.4.2 Centrifuge technique

Each time 300µL of the 1mM UMP biological media were transferred into 0.5ml MicroCentrifuge Tubes. The tubes were centrifuged for 3 min, 60µL from the bottom of the tubes were used to analyse it with the developed method. The samples got frozen with dry ice after centrifugation to stop the degradation of the Molecule.

Conc (uM)	Retention Time 1	Retention Time 2	Retention Time 3	Average	Standard deviation	relative standard deviation
1000	5,483	5,480	5,423	5,462	0,033808283	0,618972593
Conc (uM)	Height 1	Height 2	Height 3	Average	Standard deviation	relative standard deviation
1000	237.170	244.830	229617	237.206	7606,562715	3,206737352
Conc (uM)	Area 1	Area 2	Area 3	Average	Standard deviation	relative standard deviation
1000	2.954.147	3.049.527	2939491	2981055	59749,56629	2,004309424

Table 8. . HPLC results for the Filtration technic of 1mM UMP biological media with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column

The centrifugation technique attempted to be successful to filter (centrifuge) the biological medium. The centrifuge tests were reproducible in compare to the tests with the 0.2 μm syringe filters.

2.2 Stability test with UDPgal in biological medium

Stability test with UDP-Gal in biological medium, using the developed method.
UDP-Gal was dissolved in the biological medium of Varsha (RPMI 1640 medium, Gluta MAX, 2%FBS and 100 units of penicillin and 100μg of streptomycin per ml) and incubated at 37°C at a heating block (Techne Dri_Block DB-2D). Incubation time points were 1,2,3,6,24,48,72 hours. After the sample was collected it was immediately centrifuged for 3 minutes in order to separate solid material, which can block the column. At each time points two samples were tested. The first sample was directly tested in the HPLC and the second were frozen with dry Ice and tested after the first.

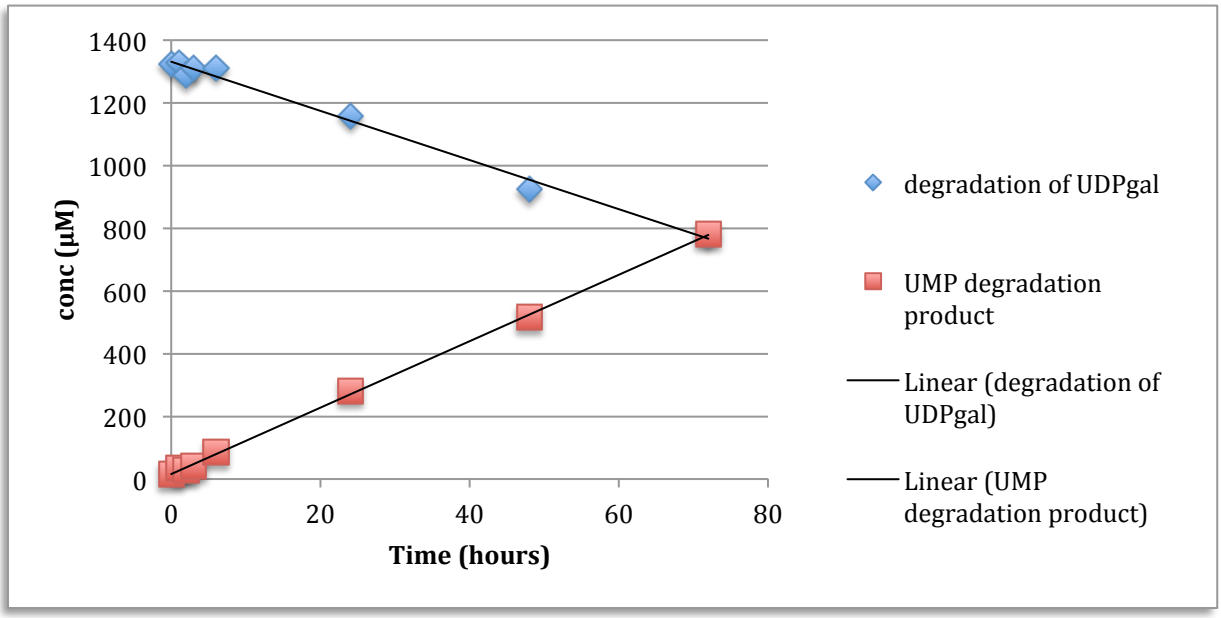


Figure 9. Stability test with UDPgal in biological medium with a Supelcosil LC – 18 –T, 5μ, 25 cm x 4.6 mm column

2.3 Stability test with UDP-Gal in water

Stability test with UDP-Gal in water, using the developed method.

UDP-Gal was dissolved in ultra pure H_2O and incubated at 37°C at a heating block (Techne Dri_Blghjock DB-2D). At each time points two samples were tested. The first sample was directly tested in the HPLC and the second were frozen with dry Ice and tested after the first.

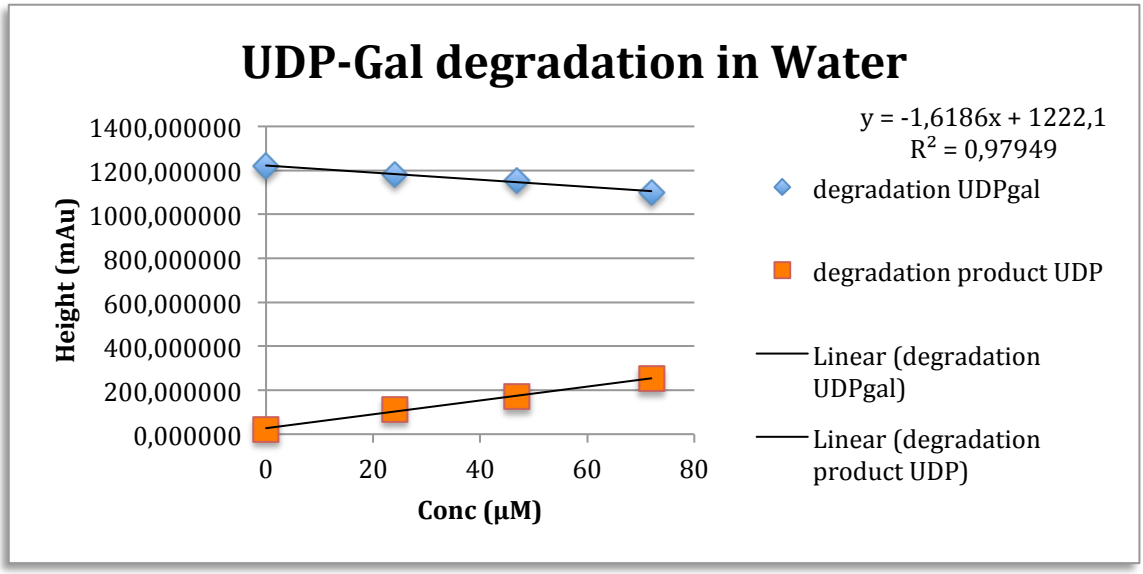


Figure 10. Stability test with UDPgal in water with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column

2.4 Stability test with UDP-Gal in brine

Stability test with UDP-Gal in brine, using the developed method.

UDP-Gal was dissolved in a 6999,5 mg/L NaCl solution and incubated at 37°C at a heating block (Techne Dri_Block DB-2D). At each time points two samples were tested. The first sample was directly tested in the HPLC and the second were frozen with dry Ice and tested after the first.

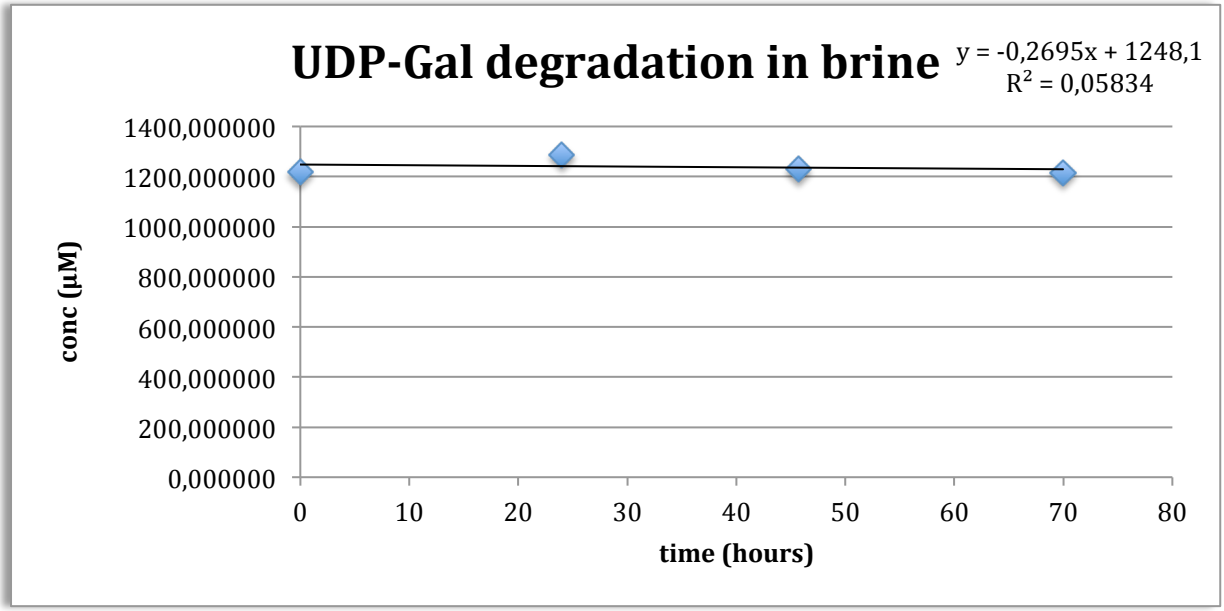


Figure 11. Stability test with UDPgal in brine with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column

2.5 Results of stability tests on UDP-Gal

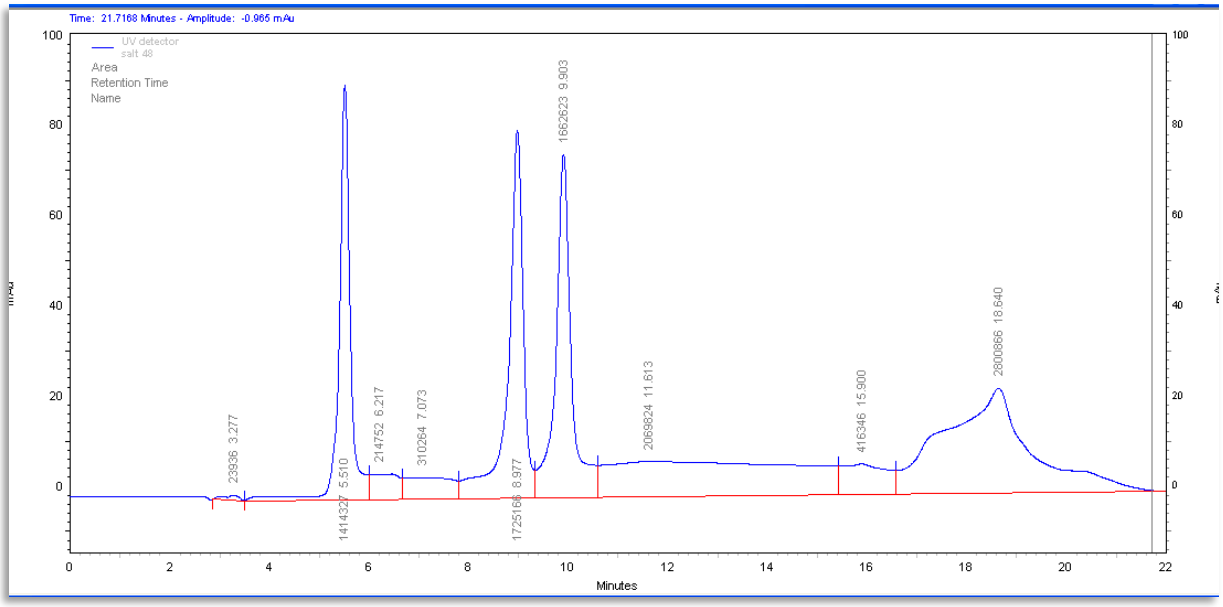
The stability of UDP-Gal was tested under various conditions and time points. UDP-Gal was decomposed in the biological medium of Varsha (RPMI 1640 medium, Gluta MAX, 2%FBS and 100 units of penicillin and 100µg of streptomycin per ml) by 12, 30, and 41% in the 24-,48- and 72 hour incubations, the main degradation product was UMP, suggesting that UDP-Gal was hydrolysed at the pyrophosphate bond. In contrast UDP-Gal was stable in brine (6999,5 mg/L NaCl) after 70hours. In Water UDP-Gal was decomposed by 3, 5, and 10% in the 24-, 47- and 72 hour incubations, different than expected UDP was the main degradation product, suggesting that UDP was hydrolysed at the phosphoester bond of the galactose.

3. Analysing the stability of UDP-GlcNAc

3.1 Methodology development

Finding a suitable method to separate the nucleotides.

According to the investigation of retention times, the developed method “Method 2” see had a good separation of the nucleotides. This method seemed suitable for the further experiments.



HPLC Graph 3. Method 2. Time point profiles of UMP, UDP and UDP-Gal with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column

2.1.3 Creating calibration a curve for UDP-GlcNAc

In order to quantify UDP-GlcNAc and its degradation products precisely calibration curves of UDP-GlcNAc, UMP and UDP must be developed “calibration curves of UDP-UMP and UDP see”.

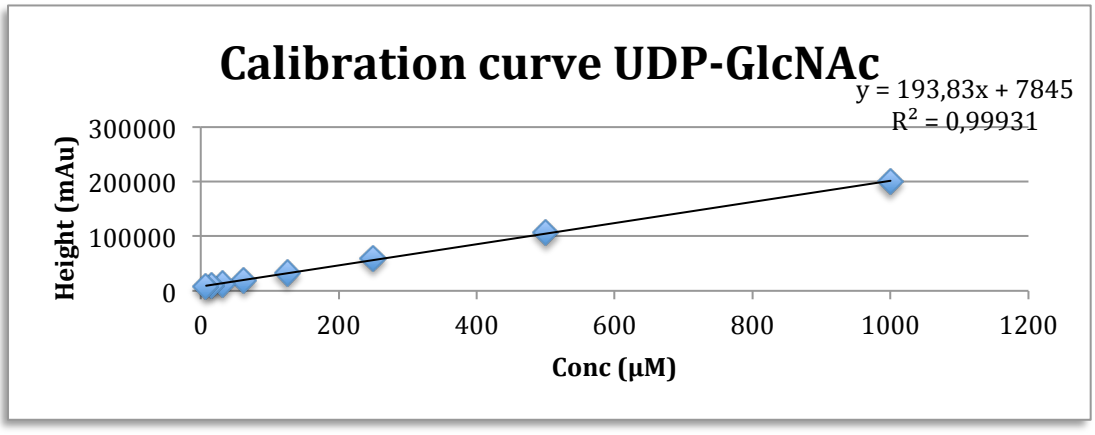


Figure 8. Calibration curve of UDP-GlcNAc with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column

3.2 Stability test with UDP-GlcNAc in biological medium

Stability test with UDP-GlcNAc in biological medium, using the developed method.
UDP-GlcNAc was dissolved in the biological medium of Varsha (RPMI 1640 medium, Gluta MAX, 2%FBS and 100 units of penicillin and 100µg of streptomycin per ml) and incubated at 37°C at a heating block (Techne Dri_Block DB-2D). Incubation time points were 0,1,2,3,24,48,72 hours. After the sample was collected it was immediately centrifuged for 3 minutes in order to separate solid material, which can block the column. At each time points two samples were tested. The samples were frozen with dry Ice and tested in the HPLC.

Figure 9. Stability test with UDPgal in biological medium with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column

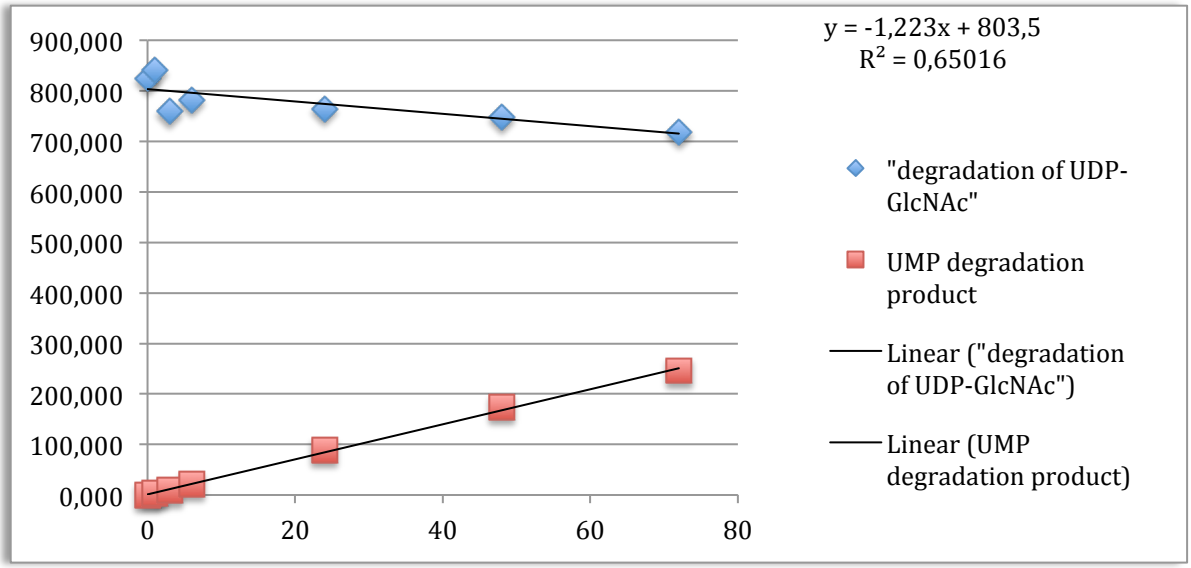


Figure 13. Stability test with UDPgal in biological medium with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column

3.3 Stability test with UDP-GlcNAc in water

Stability test with UDP-GlcNAc in water, using the developed method.
UDP-GlcNAc was dissolved in ultra pure H_2O and incubated at 37°C at a heating block (Techne Dri_Blghjock DB-2D). At each time points two samples were tested. The samples were frozen with dry Ice and tested in the HPLC.

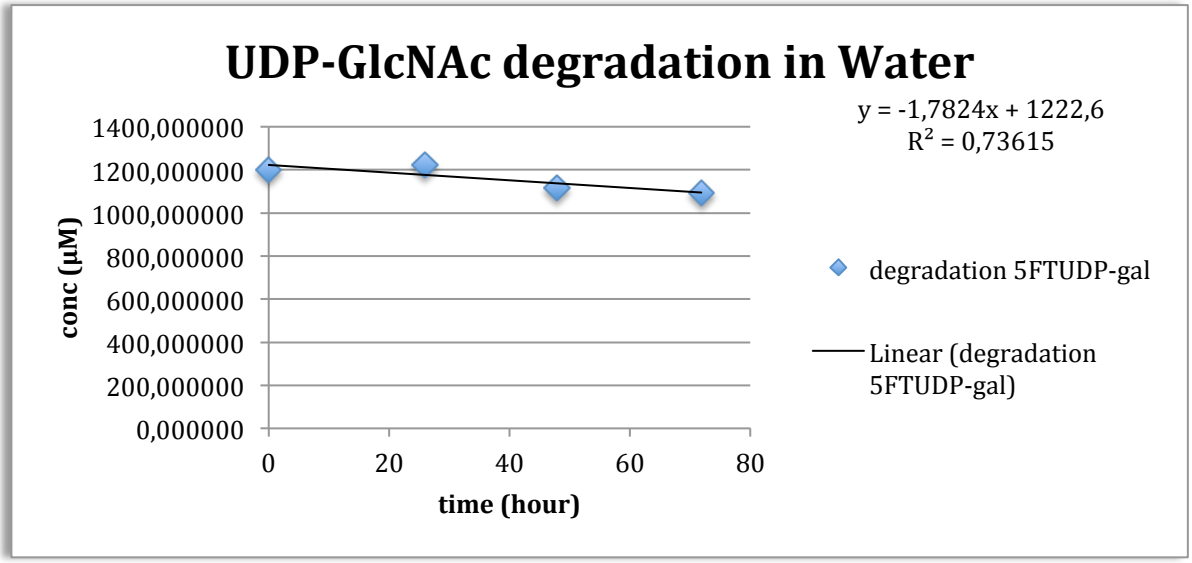


Figure 14. Stability test with UDP-GlcNAc in biological water with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column

3.4 Stability test with UDP-GlcNAc in brine

Stability test with UDP-GlcNAc in brine, using the developed method.

UDP-GlcNAc was dissolved in a 6000,0 mg/L NaCl solution and incubated at 37°C at a heating block (Techne Dri_Block DB-2D). At each time points two samples were tested. The samples were frozen with dry Ice and tested in the HPLC.

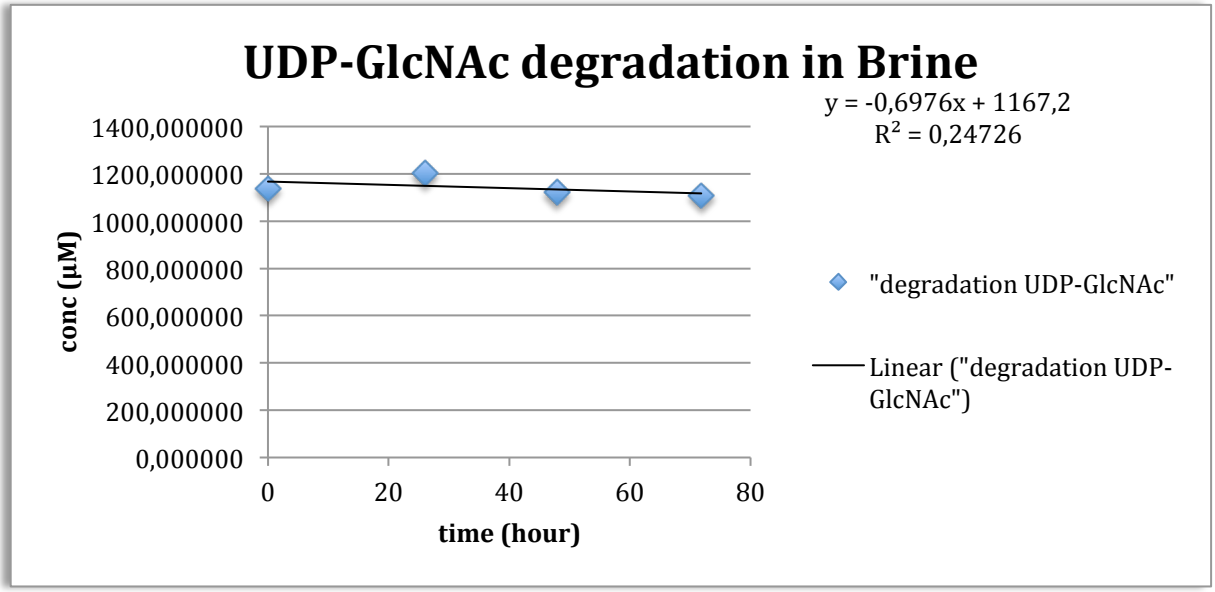


Figure 15. Stability test with UDP-GlcNAc in biological brine with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column

3.5 Results of stability tests on UDP-GlcNAc

The stability of UDP-GlcNAc was tested under various conditions and time points. UDP-GlcNAc was decomposed in the biological medium of Varsha (RPMI 1640 medium, Gluta MAX, 2%FBS and 100 units of penicillin and 100µg of streptomycin per ml) by 7, 9, and 13% in the 24-, 48- and 72 hour incubations, the main degradation product was UMP, suggesting that UDP-GlcNAc was hydrolysed at the pyrophosphate bond. In contrast UDP-GlcNAc was stable in brine (6000,0 mg/L NaCl) and water during the stability tests.

4. Analysing the stability of 5-(5-formylthien-2-yl) UDP-Gal

4.1 Methodology development

Finding a suitable method to separate the modified nucleotides.

According to the literature reverse-phase HLPC, utilizing UV detection, is the method of choice, for analysing the stability of UDP-sugars and quantify the cleavage products. The main disadvantage of this method is the long retention time for the UDP-sugars. Due to this disadvantage, different reverse-phase HPLC columns were compared with an ion exchange HPLC column.

4.1.1 Investigation of retention times of 5FT uridine diphosphate galactose, 5FTuridine diphosphate and 5FT uridine monophosphate by ion exchange HPLC.

Each of the nucleotide samples were dissolved in ultra pure H_2O, 200 µM and tested separate. Method details are shown in Table 9.

Apparatus	Aglinet 1100 Seris DAD detector		
Column	SOURCE 15Q, 6mm x 1,5 cm Hl. Glas colum		
Solvent A	Water		
Solvent B	2M NaCl		
Detection wavelength (nm)	254.8		
Reverence wavelength (nm)	Ref=700,40		
Inj. Volum	30 µL		
Flow (ml/min)	2ml/min		
Pump Program	Method 1		
	Time	A%	B%
	0	100	0
	1.5	100	0
	4.5	70	30
	5.0	50	50
	6.0	50	50
	7.0	100	0
	8.0	100	0
Retention Times Graphs are not shown.	5FTUDP-Gal - 4.024 5FTUDP- 4.500 5FTUMP - 4.088		

Table 9.HPLC conditions used for investigation of retention times of UMP, UDP and UDPgal with ion exchange colum

After the retention times of the single nucleotides were investigated, it was noticed that 5FT-UDPgal and 5FT-UMP have similar retention times. In order to find a suitable method to separate the nucleotides in the mixture 1:1:1, different methods were used. Method details are shown in Table 10.

Apparatus	Aglinet 1100 Seris DAD detector											
Column	SOURCE 15Q, 6mm x 1,5 cm Hl. Glas colum											
Solvent A	Water for method 1-3 method 4 0.1 M FTA											
Solvent B	2M NaCl											
Detection wavelength (nm)	254.8											
Reverence wavelength (nm)	Ref=700,40											
	Method 1			Method 2			Method 3			Method 4		
Inj. Volum	30 µL			30 µL			30 µL			30 µL		
	Pump Program			Pump Program			Pump Program			Pump Program		
Flow (ml/min)	T	A%	B%	T	A%	B%	T	A%	B%	T	A%	B%
2	0	100	0	0	100	0	0	100	0	0	100	0
2	1.5	100	0	1.5	100	0	1.5	100	0	1.5	100	0
2	4.5	70	30	4.5	70	30	2.5	80	20	4.5	70	30
2	5	50	50	5	50	50	42.5	70	30	5	50	50
2	6	50	50	6	50	50	43.5	50	50	6	50	50
2	7	100	0	7	100	0	45.5	50	50	7	100	0
2	8	100	0	8	100	0	46.5	100	0	8	100	0
							48	100	0			
Retention Times Graphs are not shown	Big peak at 4.074 presumably from 5FtUDPgal and 5FTUMP; 5FTUDP 4,526			Big peak at 4.089 presumably from 5FTUDPgal and 5FTUMP; 5FTUDP 4,521			The peaks of 5FTUMP and 5FTUDPgal were not good separated (overloaded) 3.619, 5FTUDP 3.844 All peaks did't separate well.			Big peak at 3.459 presumably from 5FtUDPgal and 5FTUMP; 5FTUDP 3,92. The were not good separated		

Table 10. HPLC conditions used for investigation of retention times of the mixture of 5FTUMP, 5FTUDP and 5FtUDPgal with ion exchange colum

None of the modifications attempted were successful in separating 5FtUMP from 5FtUDPgal.

4.1.2 Investigation of retention times of 5FT- uridine diphosphate galactose, 5FT-uridine diphosphate and5Ft-uridine monophosphate by reversed-phase HPLC.

Each of the nucleotide samples were dissolved in ultra pure H_2O, 200µM and tested in a 1:1:1 mixture (66 µM of each nucleotide in the mixture). Method details are shown in table 11, table 12, table 13.

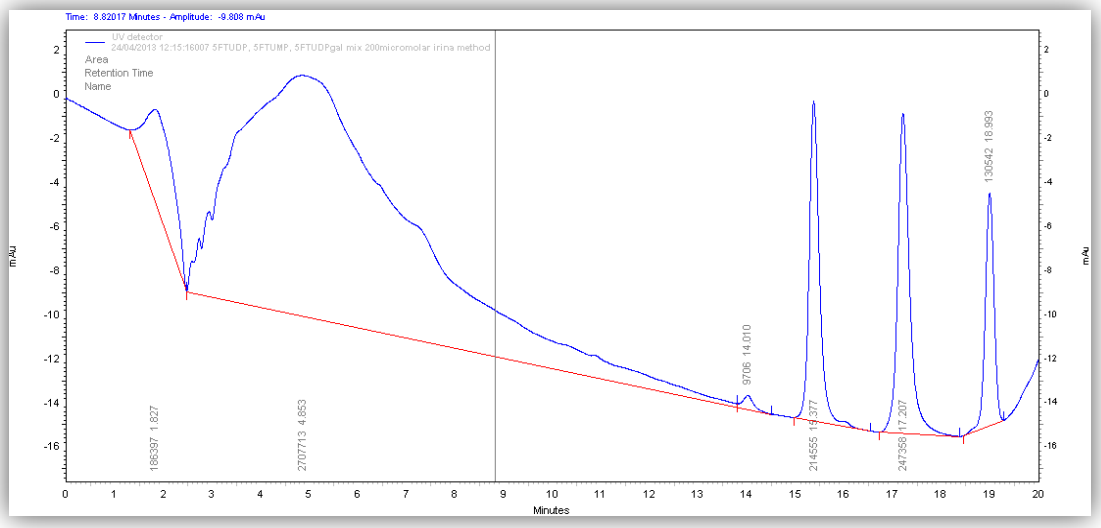
Apparatus	HPLC setup consisting of Jasco LC-Net II/ADC Interface, Jasco PU-2089 Plus Quaternary gradient pump, Jasco UV-2075 Plus Intelligent UV/Vis Detector and Jasco AS-2050 Plus Autosampler					
Column	Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column					
Solvent A	100mM Potassium Phosphate + 8mM tetrabutylammoniumhydrogensulfat pH 6.5					
Solvent B	30% MeOH +70% Solvent A					
Column Oven	Oven temperature 30.0 deg C					
Detection Wavelength (nm)	254					
	Method 1			Method 2		
Inj. Volum	30 µL			30 µL		
	Pump Program			Pump Program		
Flow (ml/min)	T	A%	B%	T	A%	B%
1	0	100	0	0	100	0
1	2	100	0	2	100	0
1	12	50	50	12	0	100
1	13	0	100	15	0	100
1	16	0	100	16	100	0
1	17	100	0	20	100	0
1	22	100	0	22	100	0
Retention Times Graphs are not shown	None of the modifications attempted were successful in separating the molecules					

Table 11. Method 1. HPLC conditions used for investigation of retention times of the mixture of 5FT-UMP, 5FT-UDP and 5FT-UDPgal with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column

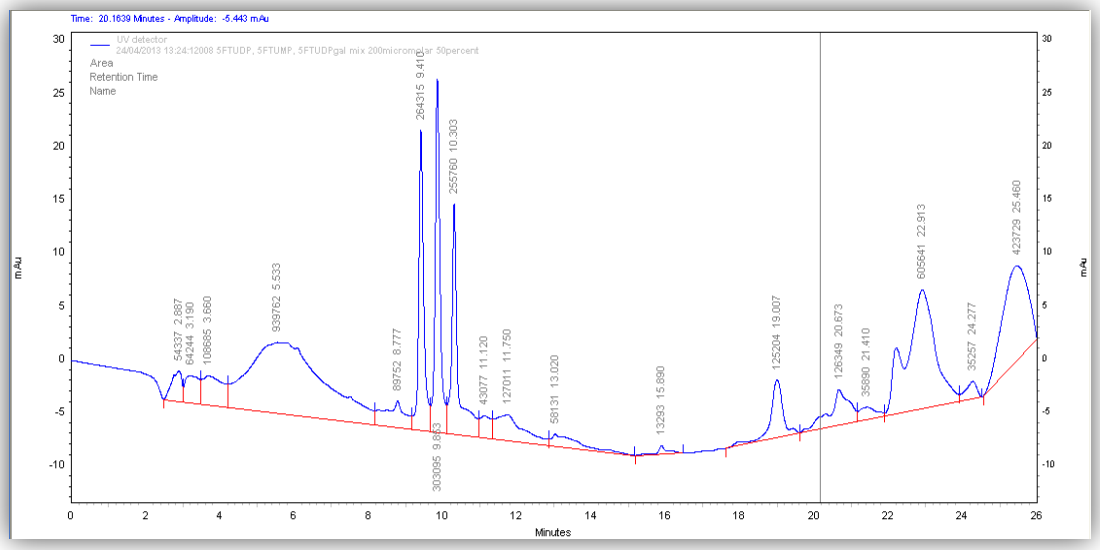
None of the modifications attempted were successful in separating the molecules.

Apparatus	HPLC setup consisting of Jasco LC-Net II/ADC Interface, Jasco PU-2089 Plus Quaternary gradient pump, Jasco UV-2075 Plus Intelligent UV/Vis Detector and Jasco AS-2050 Plus Autosampler											
Column	Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column											
Solvent A	0.05 M Phosphate buffer pH=8											
Solvent B	Methanol											
Detection wavelength (nm)	254											
Column Oven	Oven temperature 30.0 deg C											
	Method 3			Method 4			Method 5			Method 6		
Inj. Volum	µL			µL			µL			µL		
	Pump Program			Pump Program			Pump Program			Pump Program		
Flow (ml/min)	T	A%	B%	T	A%	B%	T	A%	B%	T	A%	B%
1	0	100	0	0	100	0	0	100	0	0	100	0
1	2	90	10	2	90	10	2	80	20	2	90	10
1	17	70	30	17	50	50	17	60	40	17	60	40
1	19	90	10	22	50	50	22	50	50	18	50	50
1	21	90	10	23	100	0	23	100	0	23	50	50
1	26	90	10	26	100	0	26	100	0	24	100	0
1										28	100	0
Retention Times	Details are shown in HPLC graph 3.			Details are shown in HPLC graph 4.			Details are shown in HPLC graph 5.			Details are shown in HPLC graph 6.		

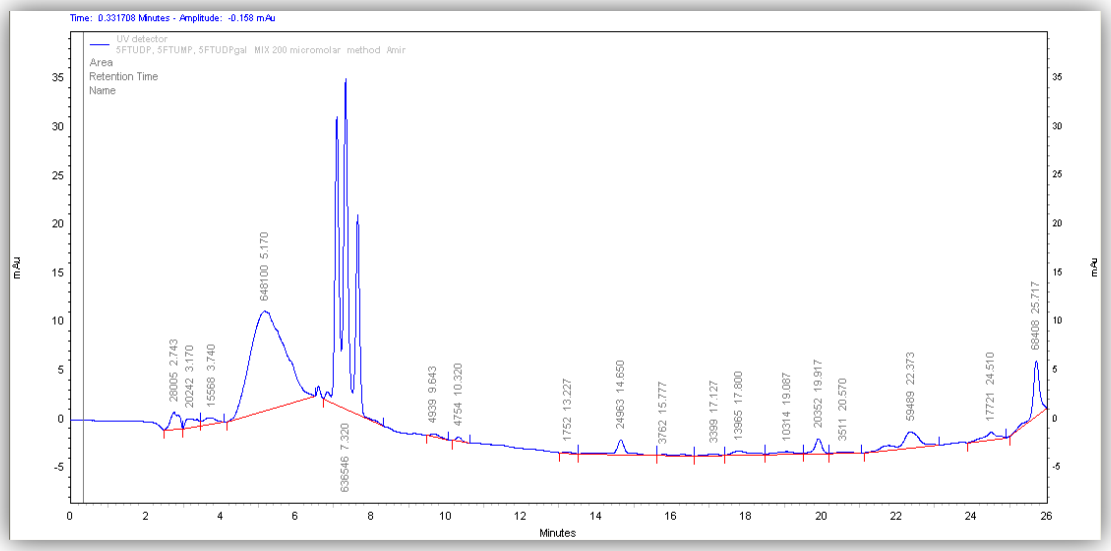
Table 12. Method 1. HPLC conditions used for investigation of retention times of the mixture of 5FT-UMP, 5FT-UDP and 5FT-UDPgal with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column



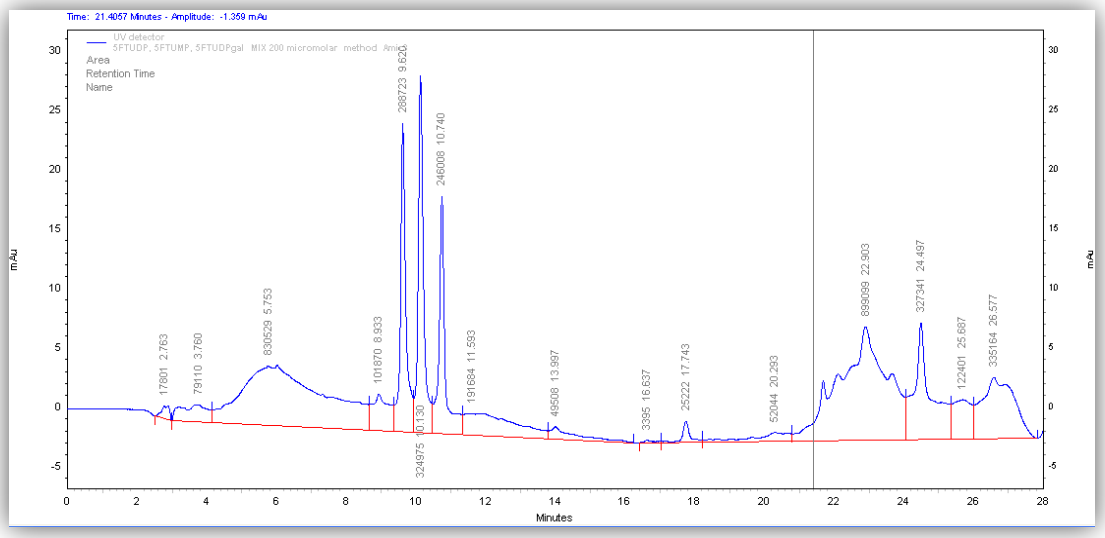
HPLC graph 3. Method 3. Time point profiles of 5FTUMP, 5FTUDP and 5FTUDP-gal with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm



HPLC graph 4. Method 4. Time point profiles of 5FTUMP, 5FTUDP and 5FTUDP-gal with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm



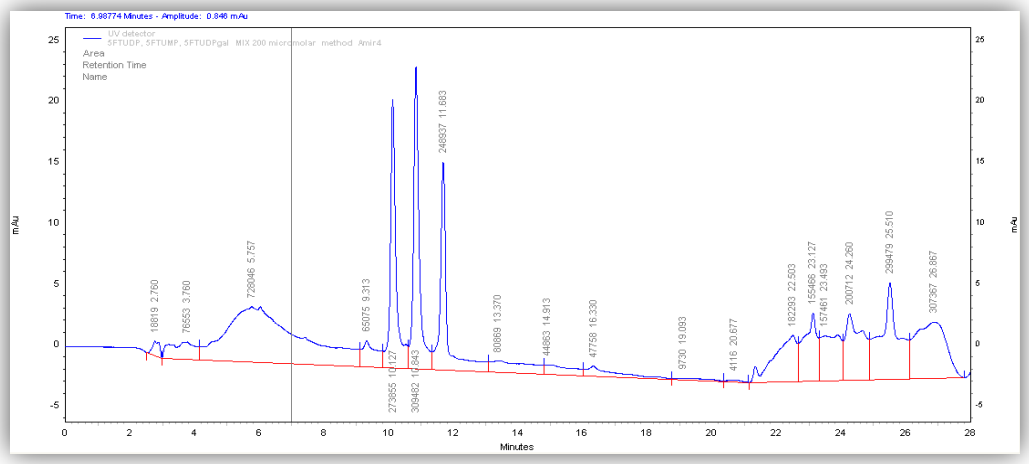
HPLC graph 5. Method 5. Time point profiles of 5FTUMP, 5FTUDP and 5FTUDP-gal with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm



HPLC graph 6. Method 6. Time point profiles of 5FTUMP, 5FTUDP and 5FTUDP-gal with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm

Apparatus	HPLC setup consisting of Jasco LC-Net II/ADC Interface, Jasco PU-2089 Plus Quaternary gradient pump, Jasco UV-2075 Plus Intelligent UV/Vis Detector and Jasco AS-2050 Plus Autosampler								
Column	Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column								
Solvent A	0.05 M Phosphate buffer pH=8								
Solvent B	Methanol								
Detection wavelength (nm)	254								
Column Oven	Oven temperature 30.0 deg C								
	Method 7			Method 8			Method 9		
Inj. Volum	30µL			30µL			30µL		
	Pump Program			Pump Program			Pump Program		
Flow (ml/min)	T	A%	B%	T	A%	B%	T	A%	B%
1	0	100	0	0	100	0	0	100	0
1	2	90	10	0.05	90	10	0.05	90	10
1	17	70	30	17	80	20	17	85	15
1	18	50	50	18	50	50	18	50	50
1	23	50	50	23	50	50	23	50	50
1	24	100	0	24	100	0	24	100	0
1	28	100	0	28	100	0	28	100	0
Retention Times	Details are shown in HPLC graph 7.			Details are shown in HPLC graph 8.			Details are shown in HPLC graph 9.		

Table 13. Method 1. HPLC conditions used for investigation of retention times of the mixture of 5FT-UMP, 5FT-UDP and 5FT-UDP-Gal with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column



HPLC graph 7. Method 6. Time point profiles of 5FTUMP, 5FTUDP and 5FTUDP-Gal with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm

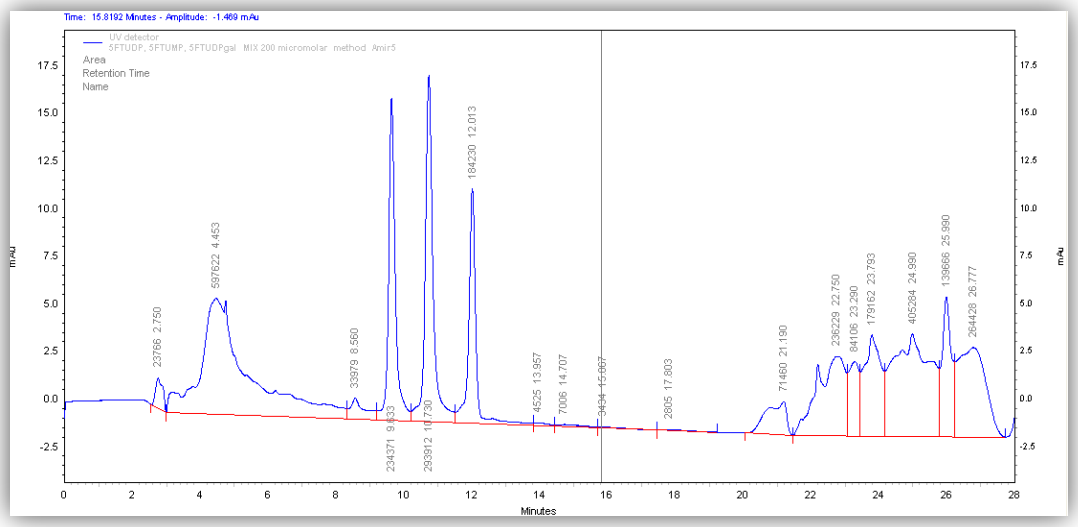


Figure 17. Method 8. Time point profiles of 5FT-UMP, 5FT-UDP and 5FT-UDP-Gal with a Supelcosil LC – 18 –T, 5µ, 25 cm

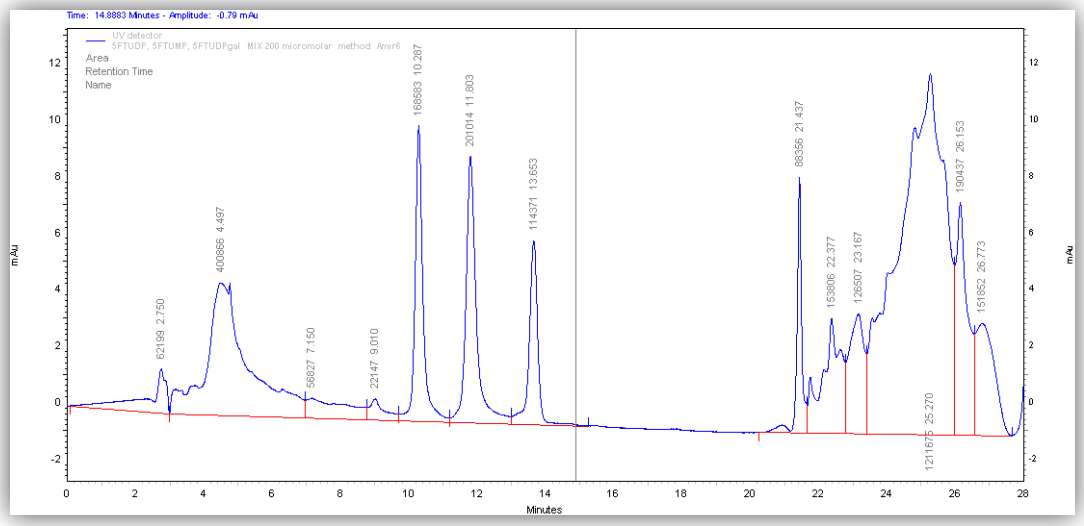


Figure 19. Method 9. Time point profiles of 5FT-UMP, 5FT-UDP and 5FT-UDP-Gal with a Supelcosil LC – 18 –T, 5µ, 25 cm

According to the investigation of retention times, the ninth method was reproducible had a good separation of the nucleotides in compare to the other methods. This method seemed suitable for the further experiments.

4.1.3 Creating calibration curves

In order to quantify 5FT-UDP-Gal and its degradation products precisely calibration curves of 5FT-UDP-Gal, 5FT-UMP and 5FT-UDP must be developed.

4.1.3.1 Calibration curve for 5FTUMP

Calibration curve for 5FTUDP based on the developed method

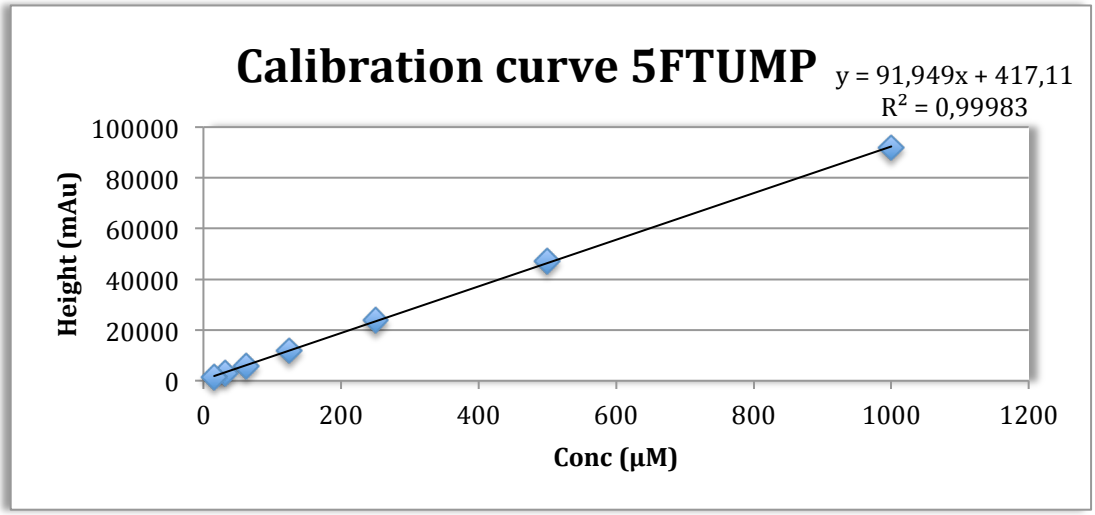


Figure 20. Calibration curve of 5FTUMP with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column

4.1.3.2 Calibration curve for 5FTUDP

Calibration curve for 5FTUDP based on the developed method

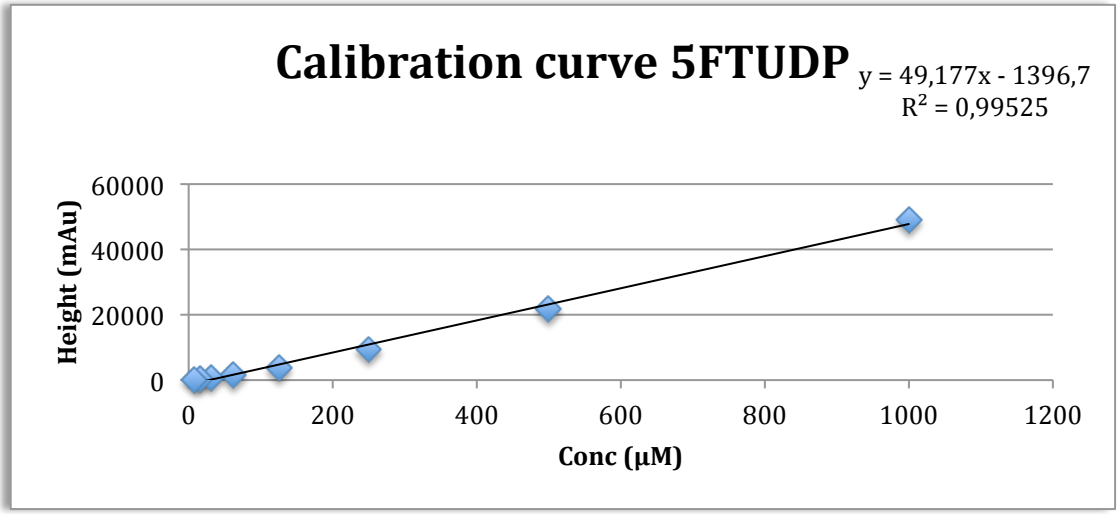


Figure 21. Calibration curve of 5FTUDP with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column

4.1.3.3 Calibration curve for 5FTUDPgal

Calibration curve for 5FTUDPgal based on the developed method

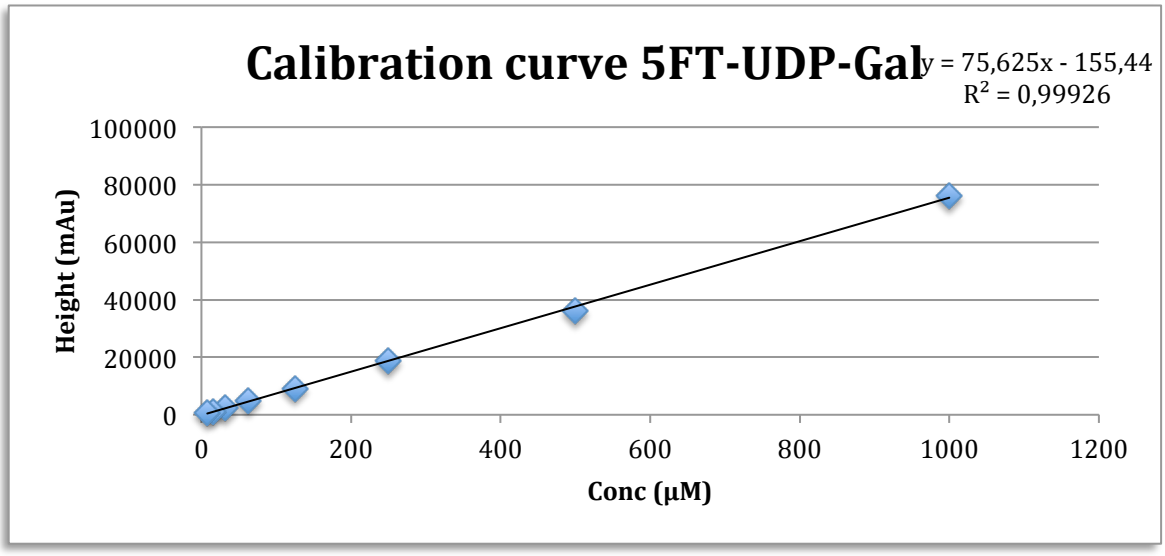


Figure 22. Calibration curve of 5FTUDPgal with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column

4.2 Stability test with 5-(5-formylthien-2-yl) UDP-Gal in biological medium

Stability test with 5FTUDP-Gal in biological medium, using the developed method. 5FTUDP-Gal was dissolved in the biological medium of Varsha (RPMI 1640 medium, Gluta MAX, 2%FBS and 100 units of penicillin and 100µg of streptomycin per ml) and incubated at 37°C at a heating block (Techne Dri_Block DB-2D). Incubation time points were 1,2,3,24,48 and 72 hours. After the sample was collected it was immediately centrifuged for 3 minutes in order to separate solid material, which can block the column. At each time points two samples were tested. The samples were frozen with dry Ice and tested in the HPLC.

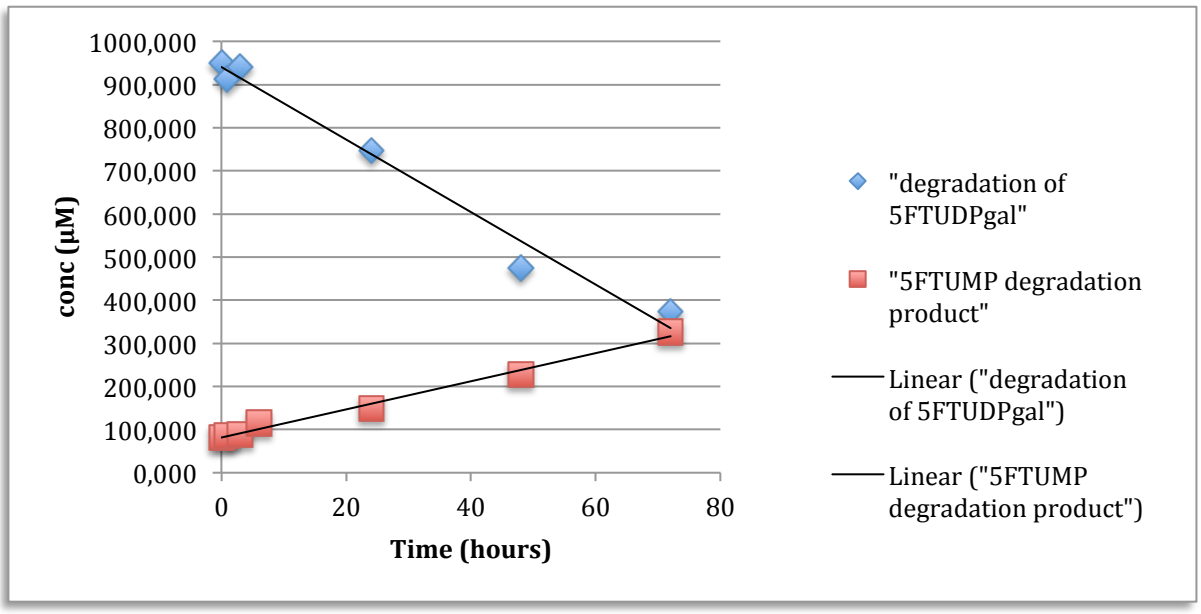


Figure 23. Stability test with 5FT-UDPgal in medium with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column

3.3 Stability test with 5-(5-formylthien-2-yl) UDP-Gal in water

Stability test with 5FTUDP-Gal in water, using the developed method.

5FTUDP-Gal was dissolved in ultra pure H_2O and incubated at 37°C at a heating block (Techne Dri_Blghjock DB-2D). At each time points two samples were tested. The samples were frozen with dry Ice and tested in the HPLC.

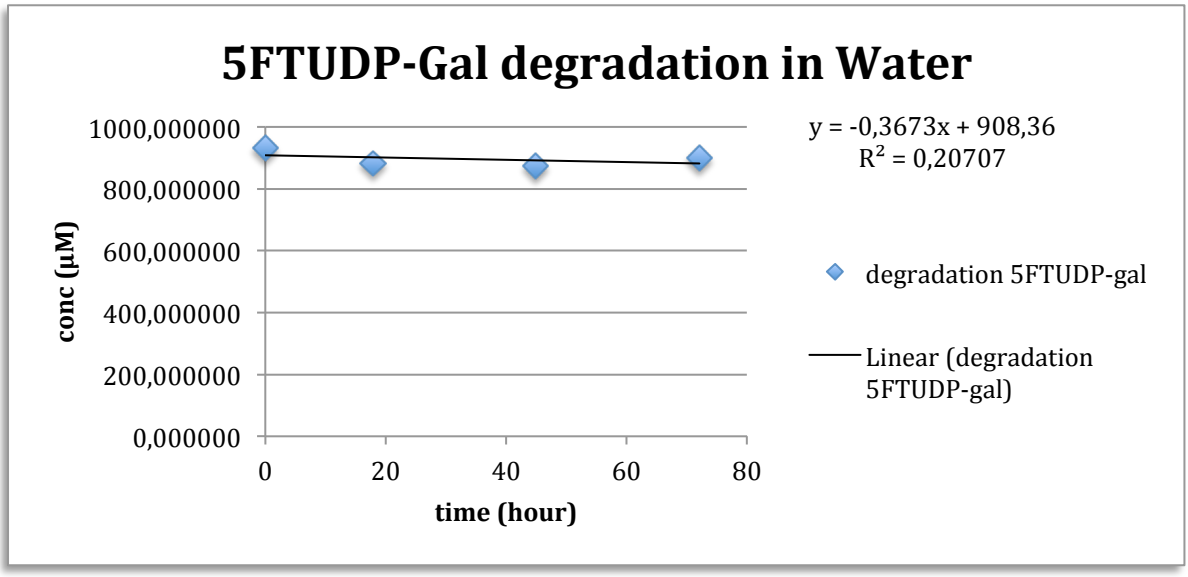


Figure 24. Stability test with 5FT-UDPgal in water with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column

4.4 Stability test with 5-(5-formylthien-2-yl) UDP-Gal in brine

Stability test with 5FTUDP-Gal in brine, using the developed method.
5FTUDP-Gal was dissolved in a 6000,0 mg/L NaCl solution and incubated at 37°C at a heating block (Techne Dri_Block DB-2D). At each time points two samples were tested. At each time points two samples were tested. The samples were frozen with dry Ice and tested in the HPLC.

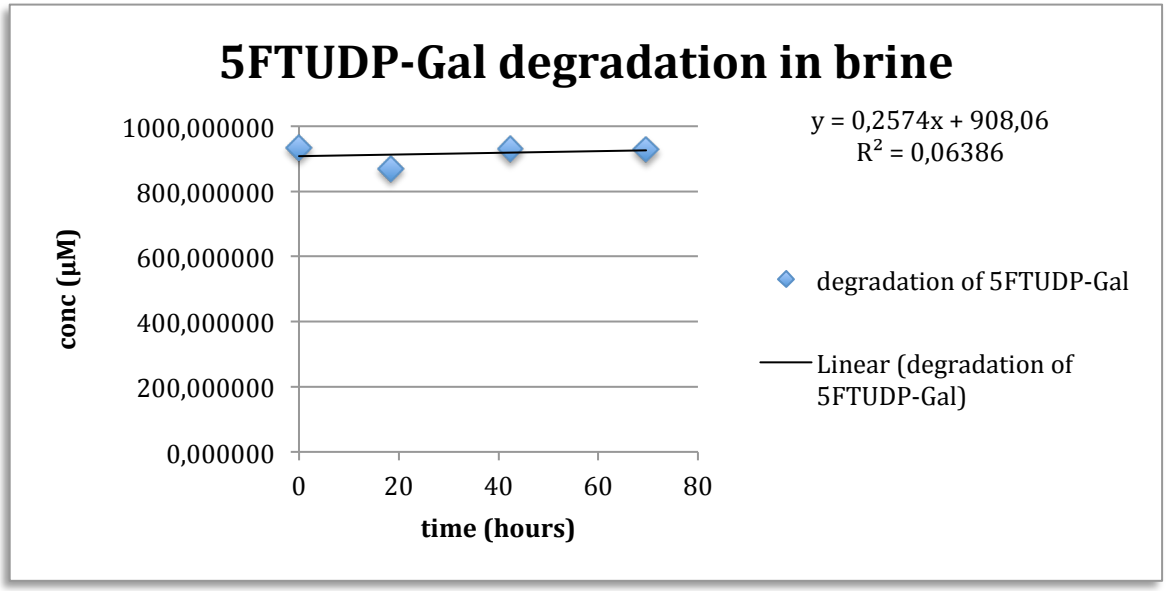


Figure 25. Stability test with 5FT-UDP-Gal in brine with a Supelcosil LC – 18 –T, 5µ, 25 cm x 4.6 mm column

4.5 Results

The stability of 5-(5-formylthien-2-yl) UDP-Gal was tested under various conditions and time points. 5FT-UDP-Gal was decomposed in the biological medium of Varsha (RPMI 1640 medium, Gluta MAX, 2%FBS and 100 units of penicillin and 100µg of streptomycin per ml) by 21, 50, and 61% in the 24-, 48- and 72 hour incubations, the main degradation product was 5FT-UMP, suggesting that 5FT-UDP-Gal was hydrolysed at the pyrophosphate bond. In contrast 5FT-UDP-Gal was stable in brine (6000,0 mg/L NaCl) and water during the stability tests.

7. List of Abbreviations

Deg C	Degree Celsius
FBS	
HPLC	High-performance liquid chromatography
UDP	Uridine diphosphate
UDP-Gal	Uridine diphosphate galactose
UDP-GalNAc	Uridine diphosphate N-Acetylgalactosamine
UDP-Glc	Uridine diphosphate glucose
UDP-GlcA	Uridine diphosphate glucuronic acid
UDP-GlcNAc	Uridine diphosphate N-Acetylglucosamin
5FT-UDP	5-(5-formylthien-2-yl) uridine diphosphate
5FT-UDPgal	5-(5-formylthien-2-yl) uridine diphosphate galactose
UMP	Uridine monophosphate
5FT-UMP	5-(5-formylthien-2-yl) uridine monophosphate
MeOH	Methanol
Mn^{2+}	$Manganese^{2+}$
$MnCl_2$	Manganese(II) chloride
NaCl	Sodium chloride
RPMI	Roswell Park Memorial Institute medium

8. References and Notes

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