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LABORATORY ANALYSIS REPORT

for

**THE HEALTH COMMISSION,
KENYA CONFERENCE OF CATHOLIC
BISHOPS
NAIROBI**

Date of Sampling: 13th November 2014
Date of Report: 16th November 2014

A handwritten signature in blue ink, appearing to be 'J. M. M.', is written over the date of report.

Table of Contents

1.0 Executive Summary Page 3

2.0 Summary of Findings..... Page 4

3.0 Introduction Page 5

4.0 Analytical Results Page 6

5.0 Interpretation of Results Page 19

6.0 List of References Page 20

1.0 EXECUTIVE SUMMARY

The Through the Catholic Health Commission of Kenya, the Catholic Church runs close to 30% of all healthcare facilities in Kenya. The Church has an expansive network which consists of 448 health units (54 hospitals, 83 health centres and 311 Dispensaries) and more than 46 Community Based Health and Orphaned and Vulnerable Children (OVC) Programs. In the Arid and Semi-Arid Areas, the Church has and manages mobile clinics for nomadic communities.

The Catholic Health commission has requested for laboratory analysis of six samples of the tetanus vaccine that was administered by the World Health Organization (WHO) / United Nations Childrens Fund (UNICEF) in the last campaign to determine the presence of human chorionic gonadotropin hormone (HCG).

The scope of the laboratory assessment was to determine the presence of human chorionic gonadotropin hormone (HCG) in all the samples collected.

1.1 OBJECTIVES OF THE LABORATORY ASSESSMENT

The Laboratory assessment had two objectives:

- 1.1.1 To assist the Catholic Health Commission in determining the presence of human chorionic gonadotropin hormone (HCG) in the samples tested.
- 1.1.2 To inform and interpret to the Catholic Health Commission the findings from the laboratory analysis

1.2 REFERENCES USED DURING THE LABORATORY ASSESSMENT

- 1.2.1 Purification of Human Chorionic Gonadotropin Hormone by Anion-Exchange High Performance Liquid Chromatography (HPLC)
- 1.2.2 Journal of Chromatography A, 847 (1999) - Determination of the molecular size distribution of type b tetanus toxoid conjugate vaccines by size exclusion chromatography

1.3 EXECUTION OF THE ASSESSMENT

The assessment was carried out at as follows:

- 1.3.1 Sampling of the samples at St. Michaels Medical Care clinic on the 13th of November 2014
- 1.3.2 Laboratory Analysis using High Performance Liquid Chromatography (HPLC) on the 14th of November 2015
- 1.3.3 Reporting and interpretation of the data on 15th November 2014

2.0 SUMMARY OF FINDINGS

Tetanus is acquired when the spores of the bacterium *Clostridium Tetani* infect a wound or the umbilical stump. Spores are universally present in the soil. People of all ages can get tetanus but the disease is particularly common and serious in newborn babies ("neonatal tetanus"). It requires treatment in a medical facility, often in a referral hospital. Neonatal tetanus, which is mostly fatal, is particularly common in rural areas where deliveries are at home without adequate sterile procedures. WHO estimated that neonatal tetanus killed about 180 000 babies in 2002.

Tetanus can be prevented through immunization with tetanus-toxoid (TT) -containing vaccines. Neonatal tetanus can be prevented by immunizing women of childbearing age with tetanus toxoid, either during pregnancy or outside of pregnancy. This protects the mother and - through a transfer of tetanus antibodies to the fetus - also her baby.

Human Chorionic Gonadotropin (HCG) hormone is synthesized by the chorionic tissue of the placenta and is found in urine during pregnancy. HCG comprises the glycoprotein hormone family. HCG is dimeric and is composed of two non-covalently bonded glycopeptides sub-units termed α (alpha HCG) and β (beta HCG).

A High Performance Liquid Chromatography (HPLC) method has been developed for the detection of HCG hormone sample in one chromatographic run using anion exchange chromatography. During the 60 minute linear gradient run, complete separation was accomplished in 40 minutes. The retention time for the HCG peak using this method was about 35 minutes.

Of the six vaccine samples collected and subjected to Laboratory HPLC analysis, the following samples were found to contain the HCG hormone:

- Sample S1 KA retention time 36.283 mins and peak area of HCG was 37%
- Sample S2 KB retention time 35.825 mins and peak area of HCG 26.5%
- Sample S3 KC retention time 36.583 mins and peak area of HCG 23.9%

The laboratory assessment has revealed that three out of the six tetanus vaccine samples analysed contain the human chorionic gonadotropin (HCG) vaccine.

3.0 INTRODUCTION

Human chorionic Gonadotropin (HCG) hormone is synthesized by the chorionic tissue and is found in urine during pregnancy.

HCG together with luteinizing hormone (LH), follicle-stimulating hormone (FSH, follotropin) and thyroid-stimulating hormone (TSH, thyrotropin) comprise the glycoprotein hormone family. All are dimeric and composed of two non-covalently bonded glycopeptide subunits termed α and β . The total number of amino acid residues in HCG for the α subunit is 92 and for the β 3 chain is 147. The amino acid sequences of both subunits of HCG have also been determined by Pierce and Morgan et al. Oligosaccharide and sialic acid chains are attached to both subunits. Each subunit is extensively crosslinked by intramolecular disulfide bonds.

A great number of immunological methods for HGC assays are available. These are based on haemagglutination, latex particle agglutination, complement fixation and radioimmuno-reaction. The sensitivity and specificity of these methods make them potentially useful for the measurement of HCG hormone in urine concentration.

High separation of HCG has been obtained by simple methods of column chromatography on diethyl-aminoethyl (DEAE) Sephadex and Sephadex G-100 columns by Bell et al. Another method employing stepwise gradient with increasing NaCl concentration was reported by Yi-Han Chang et al. The isolation of HCG using these methods would be suitable but the procedures are time consuming, the standard column chromatographic procedures taking about two [2] weeks.

A rapid isolation of HCG can be accomplished in one day using anion exchange chromatography.

3.1 Description of the Analytical Method

The isoelectric point of the HCG molecule is about 4, solutions with higher pH contain HCG molecules in anionic form.

Eluent "A" was phosphate buffer [pH=6], while eluent "B" was 0.01M phosphate buffer [pH=3]. Buffer "B" contained 0.05M sodium sulfate and 0.05M sodium hydrogen sulfate. The pH of the solution was acidic due to the acidic character of the NaHSO₄. Buffer "A" contained 0.01% v/v of "B" leading to the sulfate and hydrogen ion concentrations being 10,000 times smaller. This means that during a 40 min linear gradient both the hydrogen ion and the sulfate concentrations have been significantly increased, conditions that promote the elution and good resolution of HCG.

The retention time of the HCG peak was about 35mins.



3.2 HPLC Apparatus and Columns

3.2.1 HPLC Equipment

The analytical HPLC system used in this assessment consisted of a Shimadzu Class VP 10 system. A Dell computer connected to Shimadzu SCL 10A - system controller was used for gradient control of the two shimadzu pumps.

The preparative procedures were accomplished on Shimadzu LC-10AT Liquid Chromatograph connected to a Shimadzu 10AV UV VIS detector with a model 201 fraction collector and equipped with a 7125 sample injection valve with 1ml sample loop.

3.2.2 Column

Anion exchange separations were performed on an ODS 5µm 250mm x 4.6mm column.

3.2.3 Materials

Mobile phases contained analytical - grade sodium acetate, sodium bicarbonate, potassium chloride, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium sulfate and sodium hydrogen sulphate.

HPLC grade acetonitrile and deionized water were used for preparing the eluents. Solvents were degassed ultrasonically.

3.2.4 Gradient Programs

The chromatographic run started isocratically by pumping 100% "A" mobile phase for 20min. A linear gradient from 0% "B" to 100% "B" mobile phase was employed at a 1.0ml/min flow rate for 40 mins. The samples were dissolved in "A" eluent and injected at the beginning of the gradient run.

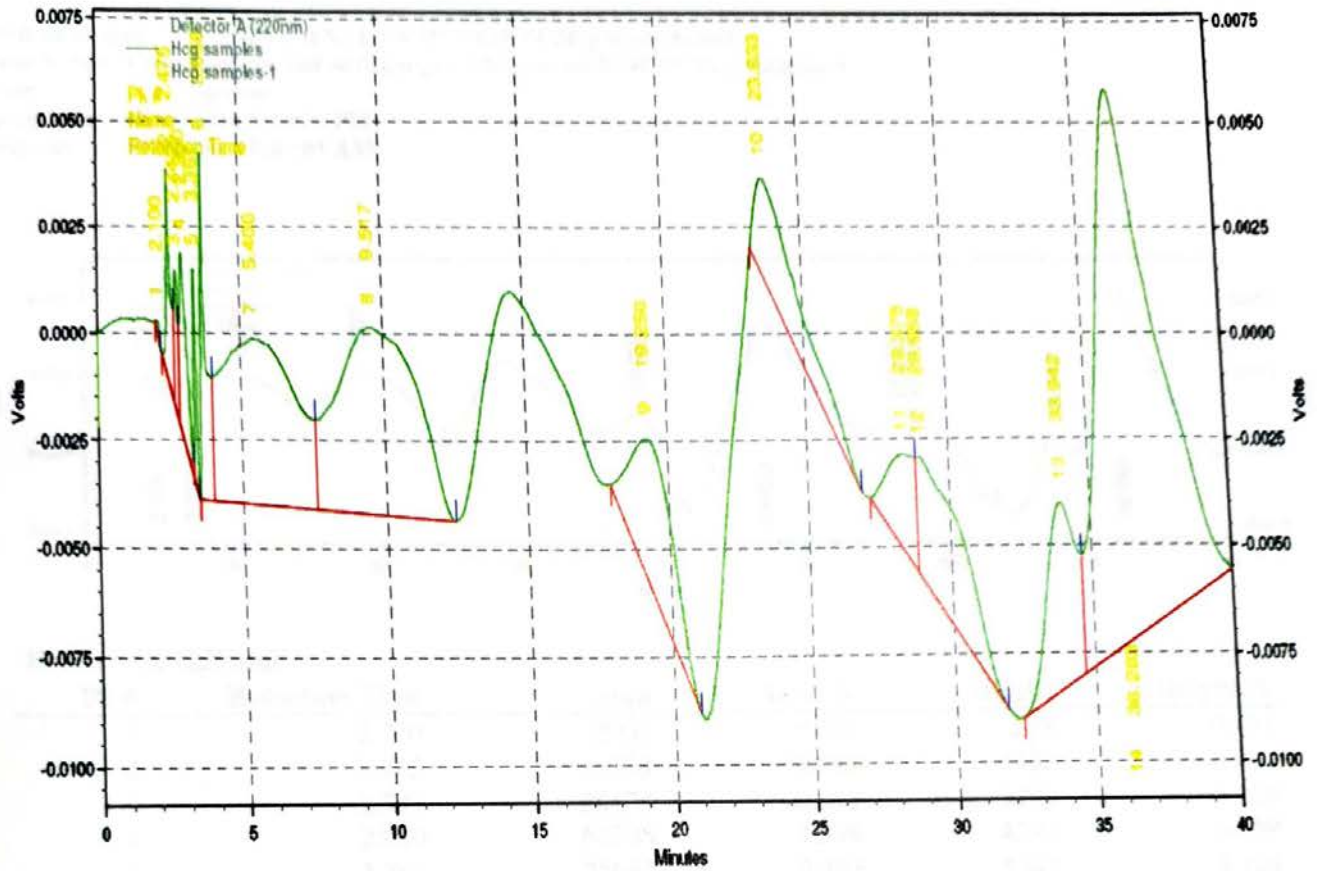
4.0 RESULTS

The samples analysed were:

- Sample 1 - KA
- Sample 2 - KB
- Sample 3 - KC
- Sample 4 - KD
- Sample 5 - KE
- Sample 6 - KF

4.1 Results Sample KA

HPLC chromatogram of Sample KA

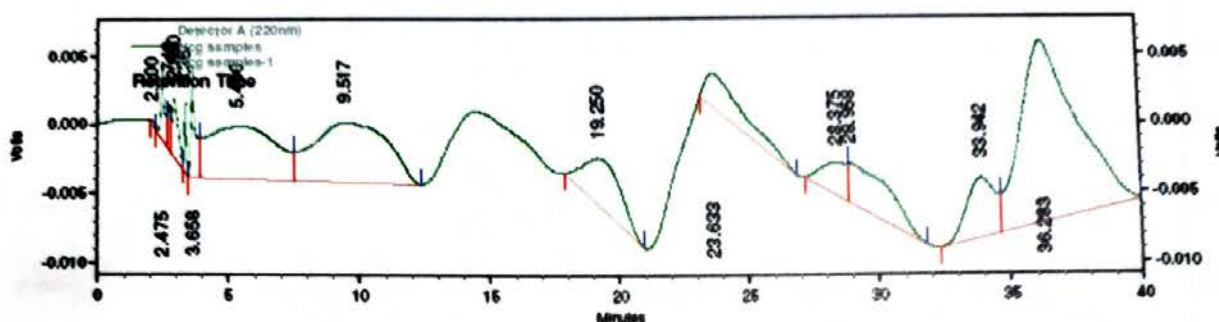


Peak area Calculation of Sample KA

Shimadzu CLASS-VP V6.12 SP4
Page 1 of 1

Area % Report

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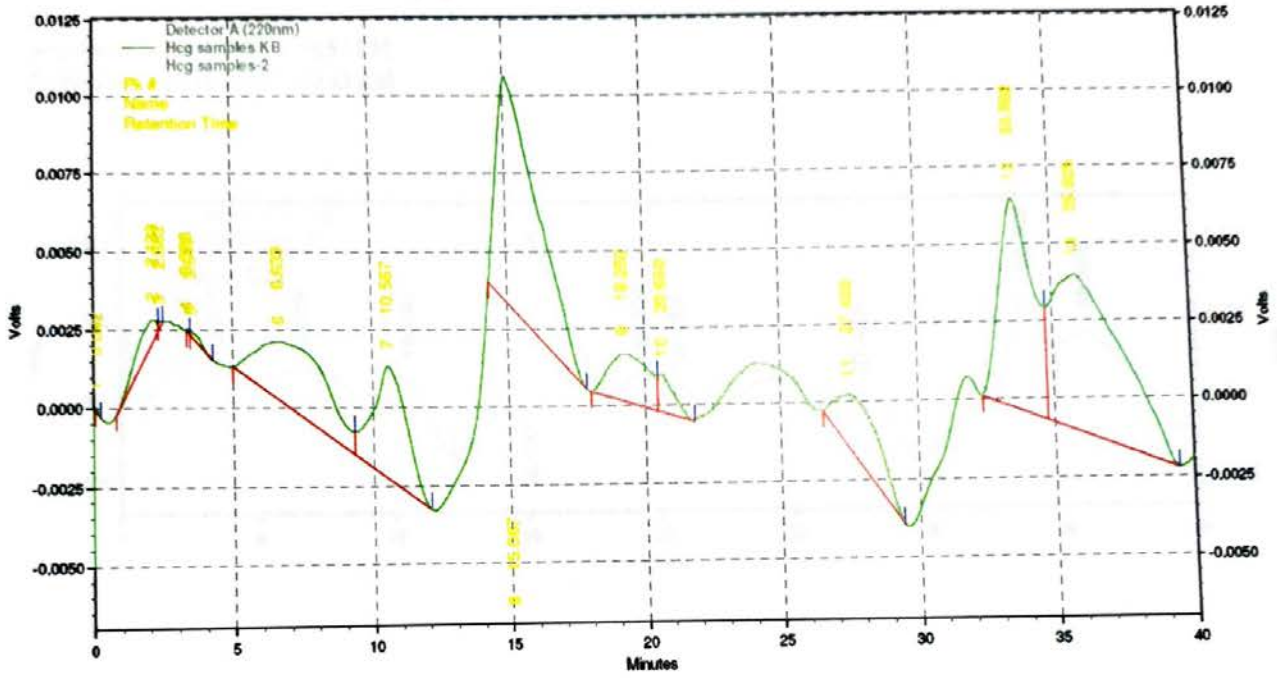
Detector A (220nm)

Pk #	Retention Time	Area	Area %	Height	Height %
1	2.100	2003	0.038	268	0.431
2	2.475	46218	0.888	4982	8.005
3	2.742	28178	0.542	3290	5.286
4	2.950	67598	1.299	4294	6.899
5	3.367	25661	0.493	5042	8.101
6	3.658	98852	1.900	8132	13.066
7	5.400	690049	13.263	3835	6.162
8	9.517	856619	16.464	4316	6.935
9	19.250	351664	6.759	3389	5.445
10	23.633	268812	5.167	2311	3.713
11	28.375	152597	2.933	2257	3.626
12	28.958	344159	6.615	2814	4.521
13	33.942	317414	6.101	4258	6.842
14	36.283	1953152	37.539	13049	20.967

Totals		5202976	100.000	62237	100.000
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4.2 Results Sample KB

HPLC chromatogram of Sample KB



Retention Time	Area	Height	Width	Volume	Height	Area
1.027	1000	100	10	1000	100	1000
2.027	2000	200	20	2000	200	2000
3.027	3000	300	30	3000	300	3000
4.027	4000	400	40	4000	400	4000
5.027	5000	500	50	5000	500	5000
6.027	6000	600	60	6000	600	6000
7.027	7000	700	70	7000	700	7000
8.027	8000	800	80	8000	800	8000
9.027	9000	900	90	9000	900	9000
10.027	10000	1000	100	10000	1000	10000
11.027	11000	1100	110	11000	1100	11000
12.027	12000	1200	120	12000	1200	12000
13.027	13000	1300	130	13000	1300	13000
14.027	14000	1400	140	14000	1400	14000
15.027	15000	1500	150	15000	1500	15000
16.027	16000	1600	160	16000	1600	16000
17.027	17000	1700	170	17000	1700	17000
18.027	18000	1800	180	18000	1800	18000
19.027	19000	1900	190	19000	1900	19000
20.027	20000	2000	200	20000	2000	20000
21.027	21000	2100	210	21000	2100	21000
22.027	22000	2200	220	22000	2200	22000
23.027	23000	2300	230	23000	2300	23000
24.027	24000	2400	240	24000	2400	24000
25.027	25000	2500	250	25000	2500	25000
26.027	26000	2600	260	26000	2600	26000
27.027	27000	2700	270	27000	2700	27000
28.027	28000	2800	280	28000	2800	28000
29.027	29000	2900	290	29000	2900	29000
30.027	30000	3000	300	30000	3000	30000
31.027	31000	3100	310	31000	3100	31000
32.027	32000	3200	320	32000	3200	32000
33.027	33000	3300	330	33000	3300	33000
34.027	34000	3400	340	34000	3400	34000
35.027	35000	3500	350	35000	3500	35000
36.027	36000	3600	360	36000	3600	36000
37.027	37000	3700	370	37000	3700	37000
38.027	38000	3800	380	38000	3800	38000
39.027	39000	3900	390	39000	3900	39000
40.027	40000	4000	400	40000	4000	40000

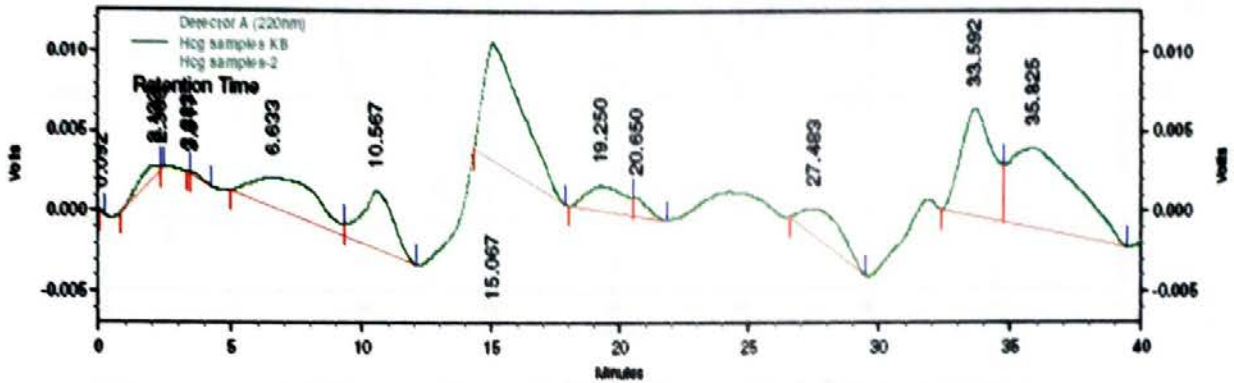
that the...
 ...not attack...
 ...responder...
 ...can also...
 ...see in the...
 ...Chan...

Peak Area Calculation Sample KB

Shimadzu CLASS-VP V6.12 SP4
Page 1 of 1

Area % Report

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Detector A (220nm)

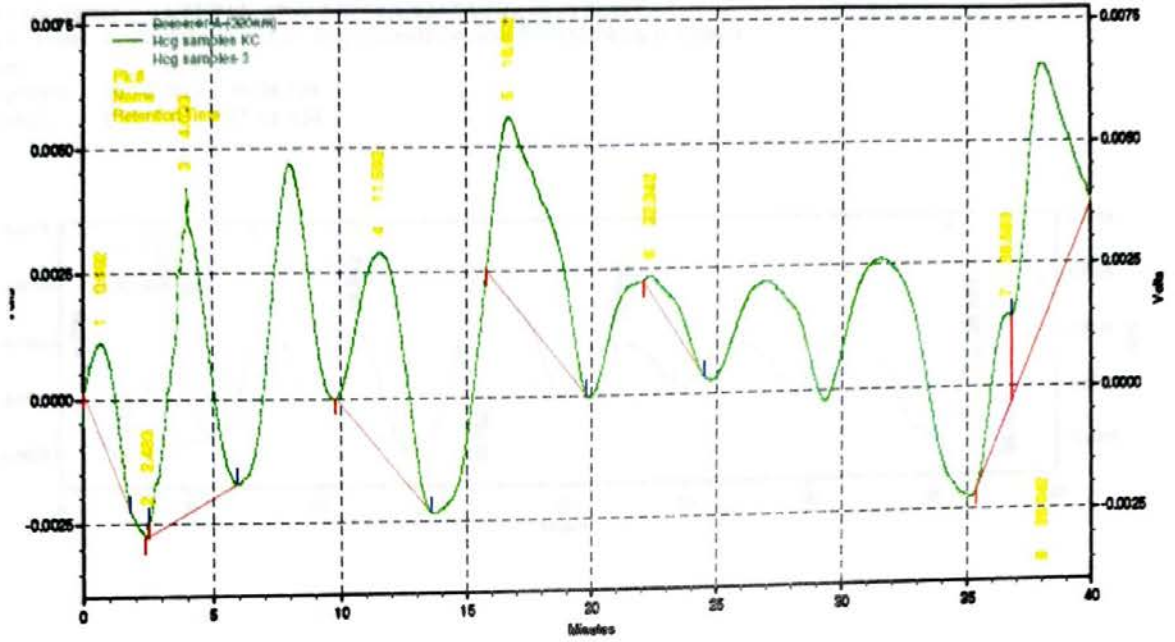
Pk #	Retention Time	Area	Area %	Height	Height %
1	0.092	646	0.019	122	0.403
2	2.133	47648	1.417	720	2.378
3	2.383	1932	0.057	242	0.799
4	3.383	635	0.019	109	0.360
5	3.517	5308	0.158	127	0.420
6	6.633	362484	10.783	1849	6.108
7	10.567	301380	8.965	3615	11.942
8	15.067	768797	22.869	7338	24.240
9	19.250	158761	4.723	1511	4.991
10	20.650	48302	1.437	1216	4.017
11	27.483	204585	6.086	1698	5.609
12	33.592	570014	16.956	6729	22.228
13	35.825	891274	26.512	4996	16.504

Totals		3361766	100.000	30272	100.000
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4.3 Results Sample KC

HPLC chromatogram of Sample KC



C:\Documents and Settings\gms\Bureaublad\UHMC\Hg samples-3, Detector A (220nm)

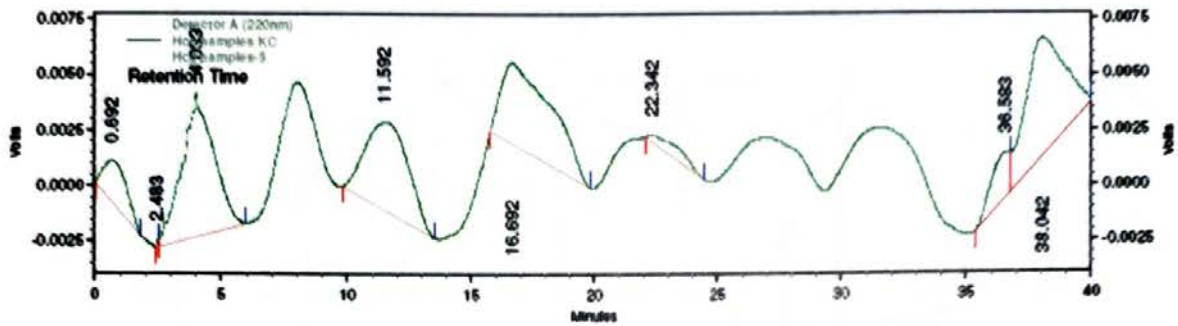


Peak Area Calculation sample KC

Shimadzu CLASS-VP V6.12 SP4
Page 1 of 1

Area % Report

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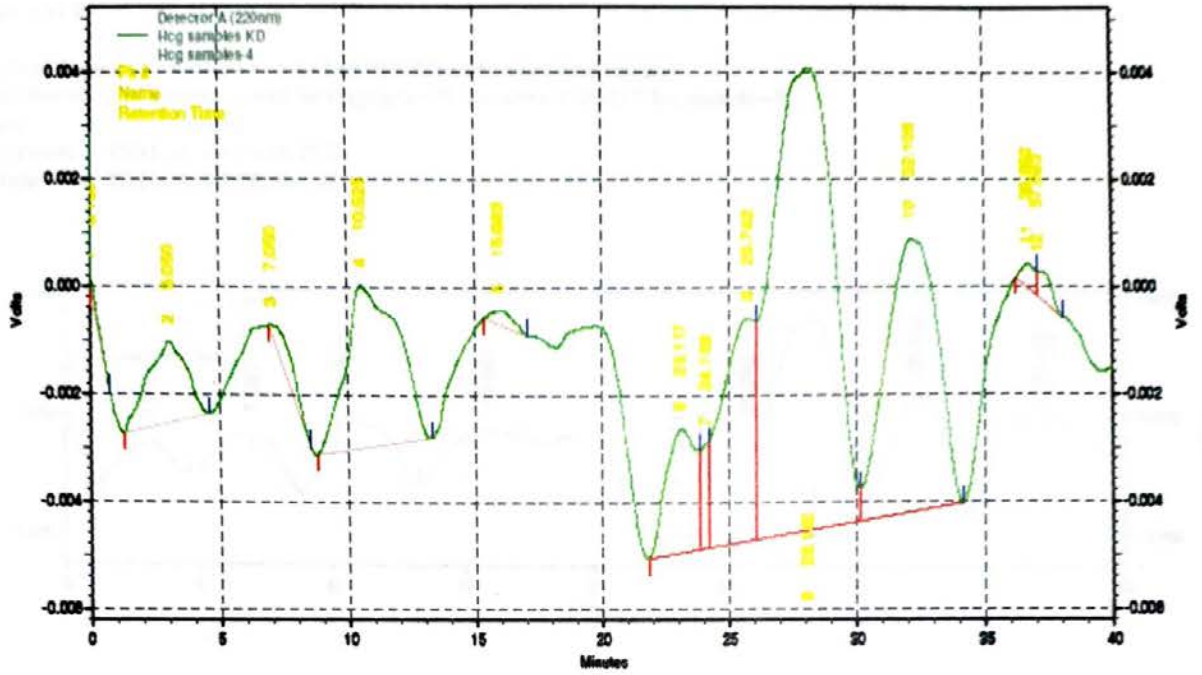
Detector A (220nm)

Pk #	Retention Time	Area	Area %	Height	Height %
1	0.692	125303	5.108	1846	7.740
2	2.483	1405	0.057	315	1.321
3	4.033	555466	22.644	6493	27.223
4	11.592	480145	19.573	3979	16.683
5	16.692	543752	22.166	3605	15.115
6	22.342	50716	2.067	328	1.375
7	36.583	109024	4.444	1993	8.356
8	38.042	587238	23.939	5292	22.188

Totals		2453049	100.000	23851	100.000
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4.4 Results Sample KD

HPLC Chromatogram of Sample KD



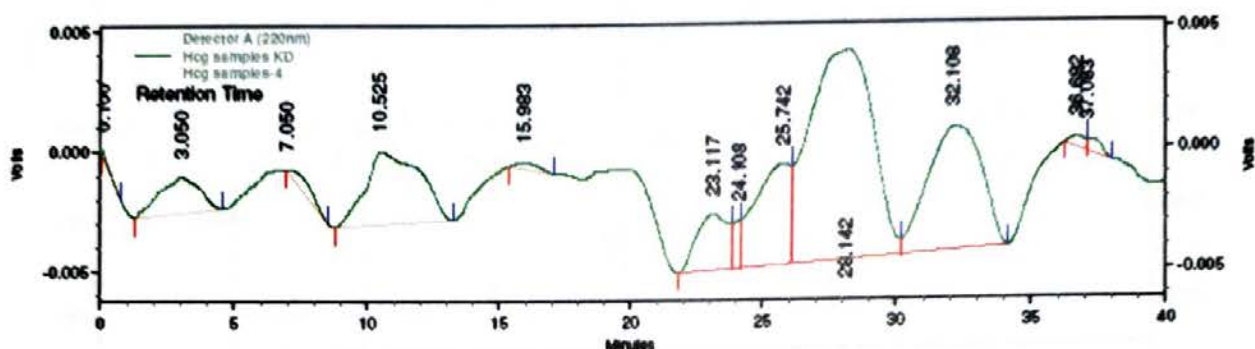
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Peak Area Calculation Sample KD

Shimadzu CLASS-VP V6.12 SP4
Page 1 of 1

Area % Report

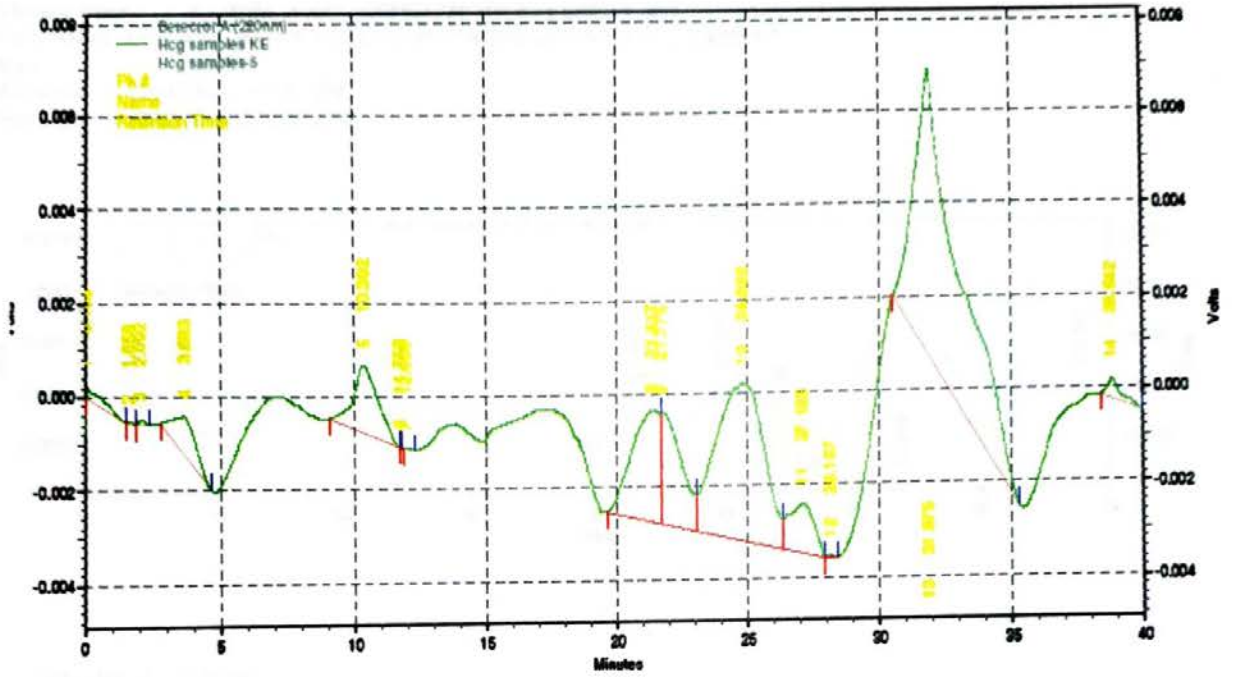
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Printed: 17/11/2014 9:48:08 AM



Detector A (220nm)						
Pk #	Retention Time	Area	Area %	Height	Height %	
1	0.100	4142	0.121	308	1.087	
2	3.050	157140	4.586	1494	5.272	
3	7.050	31315	0.914	139	0.491	
4	10.525	430352	12.560	3042	10.735	
5	15.983	16450	0.480	271	0.956	
6	23.117	186997	5.458	2321	8.190	
7	24.108	39286	1.147	1984	7.001	
8	25.742	381357	11.130	4157	14.669	
9	28.142	1435337	41.891	8633	30.464	
10	32.108	707272	20.642	5072	17.898	
11	36.692	16431	0.480	445	1.570	
12	37.083	20288	0.592	472	1.666	
Totals						
		3426367	100.000	28338	100.000	

4.5 Results Sample KE

HPLC Chromatogram Sample KE



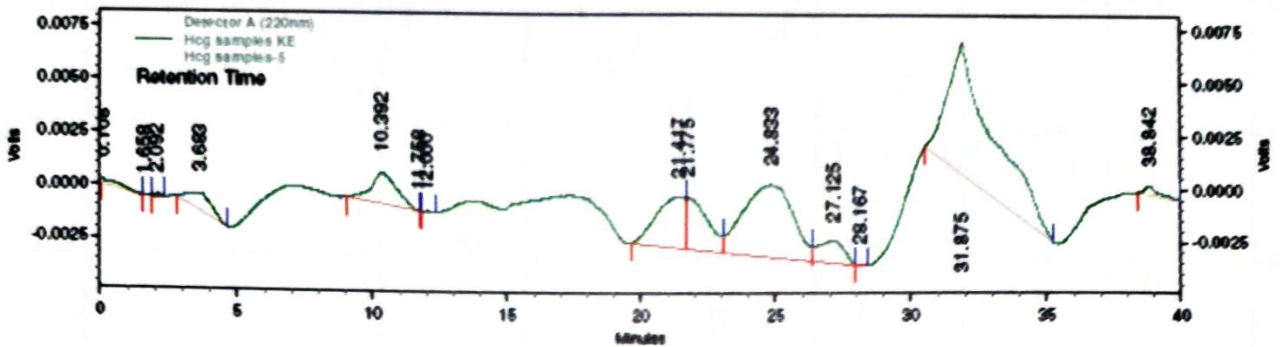
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Peak Area Calculation Sample KE

Shimadzu CLASS-VP V6.12 SP4
Page 1 of 1

Area % Report

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Data Name: C:\Documents and Settings\gcms\Bureau\blad\UHMCHcg samples-5
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Printed: 17/11/2014 9:48:35 AM



Detector A (220nm)

Pk #	Retention Time	Area	Area %	Height	Height %
1	0.108	10778	0.638	238	1.276
2	1.658	380	0.022	44	0.236
3	2.092	1347	0.080	103	0.552
4	3.683	46830	2.770	836	4.483
5	10.392	94390	5.583	1447	7.760
6	11.758	155	0.009	41	0.220
7	12.000	397	0.023	31	0.166
8	21.417	176487	10.439	2410	12.924
9	21.775	131857	7.799	2404	12.892
10	24.833	433107	25.618	3393	18.196
11	27.125	68193	4.034	1057	5.668
12	28.167	599	0.035	43	0.231
13	31.875	712097	42.120	6181	33.147
14	38.842	14035	0.830	419	2.247

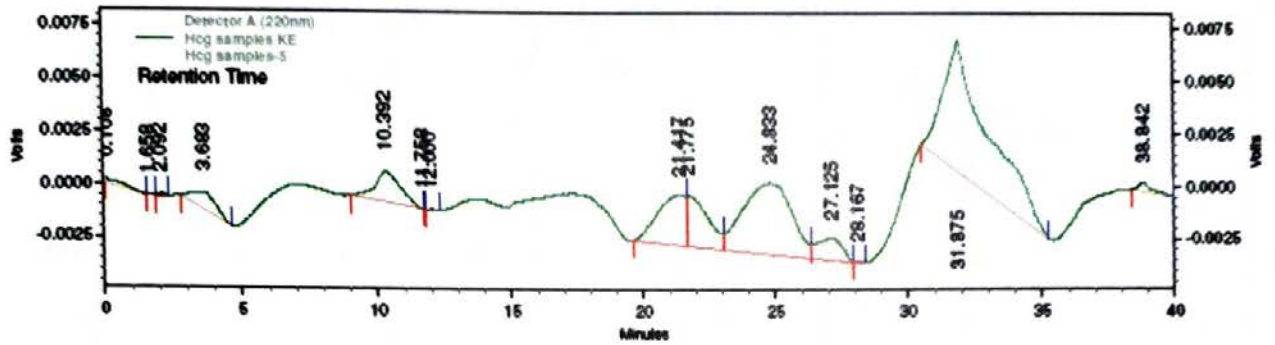
Totals		1690652	100.000	18647	100.000
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Peak Area Calculation Sample KE

Shimadzu CLASS-VP V6.12 SP4
Page 1 of 1

Area % Report

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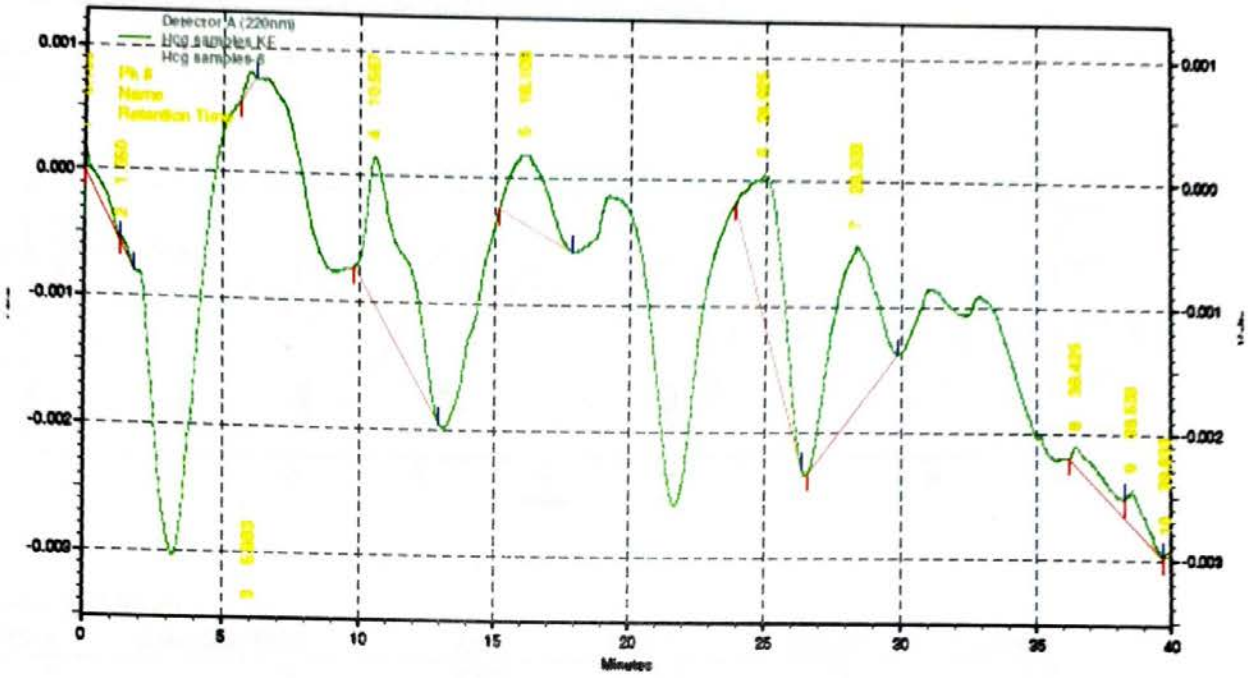


Detector A (220nm)

Pk #	Retention Time	Area	Area %	Height	Height %
1	0.108	10778	0.638	238	1.276
2	1.658	380	0.022	44	0.236
3	2.092	1347	0.080	103	0.552
4	3.683	46830	2.770	836	4.483
5	10.392	94390	5.583	1447	7.760
6	11.758	155	0.009	41	0.220
7	12.000	397	0.023	31	0.166
8	21.417	176487	10.439	2410	12.924
9	21.775	131857	7.799	2404	12.892
10	24.833	433107	25.618	3393	18.196
11	27.125	68193	4.034	1057	5.668
12	28.167	599	0.035	43	0.231
13	31.875	712097	42.120	6181	33.147
14	38.842	14035	0.830	419	2.247
Totals		1690652	100.000	18647	100.000

4.6 Results Sample KF

HPLC Chromatogram Sample KF



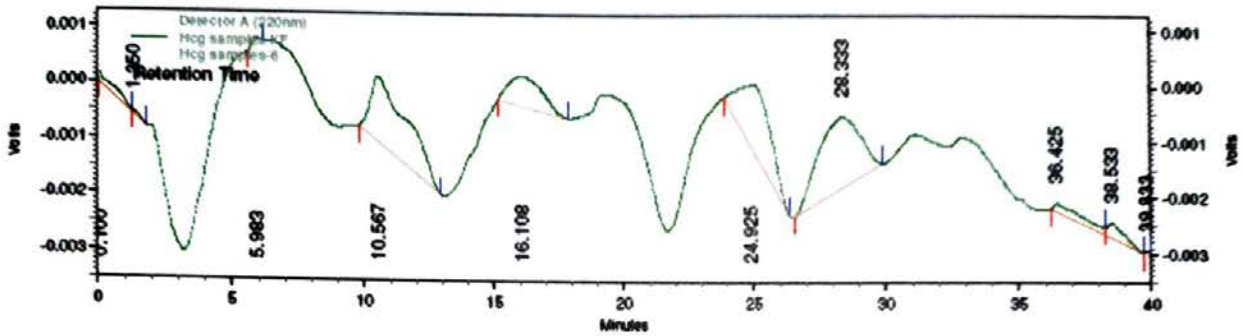
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Peak Area Calculation Sample KF

Shimadzu CLASS-VP V6.12 SP4
Page 1 of 1

Area % Report

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Printed: 17/11/2014 9:48:55 AM



Detector A (220nm)

Pk #	Retention Time	Area	Area %	Height	Height %
1	0.100	8348	1.838	191	3.825
2	1.350	1196	0.263	50	1.001
3	5.983	2420	0.533	129	2.583
4	10.567	120804	26.597	1161	23.248
5	16.108	49541	10.907	549	10.993
6	24.925	101718	22.395	1152	23.068
7	28.333	142448	31.362	1296	25.951
8	36.425	15143	3.334	151	3.024
9	38.533	12242	2.695	282	5.647
10	39.833	339	0.075	33	0.661

Totals		454199	100.000	4994	100.000

5.0 INTERPRETATION OF LABORATORY ANALYTICAL RESULTS

Following the Laboratory assessment, the following samples were found to contain human chorionic gonadotropin (HCG):

- Sample KA retention time (RT) 36.283 and peak area of 37.539%
- Sample KB retention time (RT) 35.825 and peak area 26.512%
- Sample KC retention time (RT) 38.042, peak area 23.939

All the other samples analysed did not exhibit significant elution after 35 mins and the samples could be free from HCG.