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Original Article

BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF HCG (HUMAN CHORIONIC GONADOTROPIN)

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ABSTRACT

Objective: To develop and validate simple, rapid, specific, accurate and precise bioanalytical method for determination of the HCG (Human Chorionic Gonadotropin) by RP-HPLC method by using human urine.

Methods: The chromatographic separation was performed using Phenom enex C18 (250 x 4.6 mm, 5µ, 300 °A) column. Mobile phase composed of sodium phosphate buffer (pH 7.0, 0.05M) and acetonitrile (87.5:12.5 % v/v) at a flow rate of 1 ml/min. Detection was carried out using UV detector at 215 nm. Bovine serum albumin (BSA) was used as an internal standard (ISTD) and extraction was carried out using protein precipitation method. The method was validated as per USFDA guidelines.

Results: The method was linear over the concentration range of 0.37 to 48.4 µg/ml. and correlation coefficient (R^2) was found to be 0.9983 and the Lower limit of quantitation (LLOQ) was 0.37 µg/ml. Recovery was found more than 94.0% for HCG. The % CV for interday and intraday precision was found to be less than <1%.

Conclusion: A simple, rapid, specific, accurate and precise analytical method was developed and validated by using human urine.

Keywords: RP-HPLC, HCG, BSA, Bioanalytical, Validation.

INTRODUCTION

Human chorionic gonadotropin (HCG) is a peptide or gonadotropin hormone produced in pregnancy that is made by the embryo soon after conception and later by the syncytiotrophoblast (part of the placenta). Its IUPAC name is (2S)-2-amino-6-[[(2R)-1-(carboxymethylamino)-1-oxo-3-sulfanylpropan-2-yl] amino]-6-oxohexanoic acid and it is a water soluble hormone. It consists of 237 amino acids; with an A chain containing 92 amino acids and B chain containing 145 amino acid. Its role is to prevent the disintegration of the corpus luteum of the ovary and thereby maintain progesterone production that is critical for a pregnancy in humans. Therapeutically it is mainly used in male infertility due to hypogonadotrophic hypogonadism, delayed puberty due to hypogonadism in the male and an ovulatory infertility. Diagnostically it is detected during pregnancy qualitatively [1, 2]. HPLC, LC-MS and LC-MS/MS methods are widely used for bioanalytical purpose as well as to determine drug in pharmaceutical dosage form [3, 5]. Literature reviews suggest that their enzyme based immunoassay methods available for determination of HCG in bio samples. However, these methods have some limitations which are minimized by HPLC based methods. HPLC method has following advantages over immunoassay method:

1. High specificity, at low concentration.
2. Internal standards (BSA), correct matrix effects and hence improves accuracy.
3. Wide range of the calibration curve available.
4. Lower inter-laboratory variability and lower variability among different immunoassay kits [4].

Hence present research work describes the simple, rapid, accurate, sensitive and reproducible RP-HPLC based bioanalytical method for determination of HCG in urine.

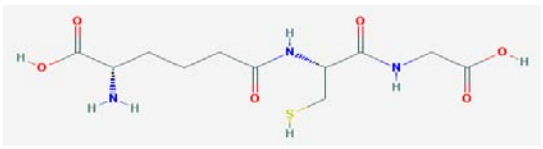

Fig. 1: Structure of HCG [1]

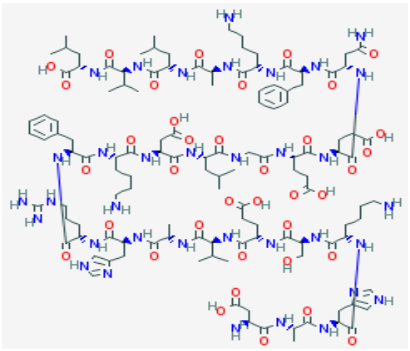

Fig. 2: Structure of BSA [1]

MATERIALS AND METHODS

Instrument and analytical conditions

The HPLC analysis was carried out using shimadzu (LC-20AD) binary gradient pump with UV detector and running as spin chrome software. The column used was Phenom enex C18 (250 x 4.6 mm, 5µ, 300 °A) column at the flow rate as 1 ml/min. and detection was performed as 215 nm. The injection volume of the sample was 20 µl. and the mobile phase was made of sodium phosphate buffer (pH 7.0, 0.05M) and acetonitrile (87.5:12.5 % v/v). The mobile phase was filtered through 0.45 µm membrane filter and sonicated before use.

Reagents and chemicals

Triple distilled water was used for the HPLC experiment. HPLC grade acetonitrile, HCG and other reagents were commercially purchased and were of AR grade.

Preparation of mobile phase

Accurately weighed 1.8 gm of disodium hydrogen phosphate and 1.5 gm sodium dihydrogen phosphate were separately transferred into 250 ml volumetric flasks and finally volume was adjusted with triple distilled water, and then degassed through sonicator. This solution

was prepared as 0.05M and pH adjusted 7.0 with ortho phosphoric acid solution. Sodium phosphate buffer and acetonitrile take in the ratio of (87.5:12.5) into the mobile phase bottle. Then filtered through 0.45µ nylon membrane filter and degassed. Mobile phase was used as diluents.

Preparation of sample and dilution pattern for HCG using urine as a matrix

Standard stock solution was prepared by dissolving 100 mg of HCG transferred into 100 ml volumetric flasks and adjusted with diluents. A series of its working solutions were prepared in order to make the concentration of 0.37, 0.74, 2.42, 7.2, 24.2, 36.3, 43.5 and 48.4 µg/ml in each 500 µl normal human urine and 1000 µl acetone. The internal standard was prepared having a concentration 24.2 µg/ml in diluents. All above solution were spiked with 24.2 µg/ml working internal standard solution and vortexes for 30 second before extraction. Extraction was done using acetone.

Chromatographic conditions

Chromatographic analysis was performed on a Phenomenex C18 (250 x 4.6 mm, 5µ, 300 °A) column. The mobile phase consists of sodium phosphate buffer: acetonitrile (87.5:12.5% v/v). The mobile phase was filtered through the membrane filter and degassed before pumping into HPLC system.

Mobile phase: Sodium phosphate buffer: water (87.5:12.5% v/v).

Flow rate: 1 ml/min.

Detection wavelength: 215 nm.

Injected volume: 20 µl.

Column temperature: Ambient.

Method validation

The developed chromatographic method was validated for system suitability, specificity, linearity and range, accuracy, precision, recovery, and stability as per USFDA guidelines [9-11].

RESULTS AND DISCUSSION

Method development and optimization

To optimize the HPLC methods, several mobile phase composition was tried. A satisfactory peak separation, resolution, theoretical plate and tailing factor for both drug and internal standard were found good with mobile phase sodium phosphate buffer (pH 7.0, 0.05M): acetonitrile (87.5:12.5 % v/v) at a flow rate of 1 ml/min. Standard solution of HCG was scanned over the range of 200-400 nm and the wavelength was selected according to reported wavelength 215 nm. Retention time was observed as 5.279 min. and 2.343 min. for HCG and internal standard (BSA) respectively.

System suitability

System suitability was checked of a system to ensure system performance before or during the analysis of unknowns. Parameters such as theoretical plate count, tailing factors, resolution and reproducibility in retention time (RT) for six repetitions of 100% concentration of HCG and BSA was also explored to comply the system suitability test. The result of system suitability is shown in table: 1 and table: 2.

Table 1: System suitability parameters of developed bioanalytical method

S. No.	Retention time		Tailing factor		Resolution		Theoretical plate	
n=6	HCG	ISTD	HCG	ISTD	HCG	ISTD	HCG	ISTD
1.	5.278	2.341	1.152	1.225	13.350	0.00	3262	2146
2.	5.279	2.340	1.151	1.223	13.410	0.00	3207	2132
3.	5.280	2.345	1.150	1.224	13.370	0.00	3277	2179
4.	5.281	2.342	1.151	1.226	13.267	0.00	3254	2150
5.	5.279	2.343	1.150	1.225	13.432	0.00	3289	2194
6	5.279	2.344	1.152	1.226	13.290	0.00	3227	2162

Specificity

Specificity was checked for interference of the matrix in the analysis of injecting the sample solution under optimized chromatographic condition. It contains one blank, one double blank and one standard peak. The peaks purity was found to be good and no interference was found for both drug and ISTD. Percentage coefficient of variation (%CV) of retention time was found 9.47% for HCG and 0.040% for ISTD which are within the acceptance range hence it could be said that the developed method was highly specific. The result of specificity is shown in the table: 3 and table: 4.

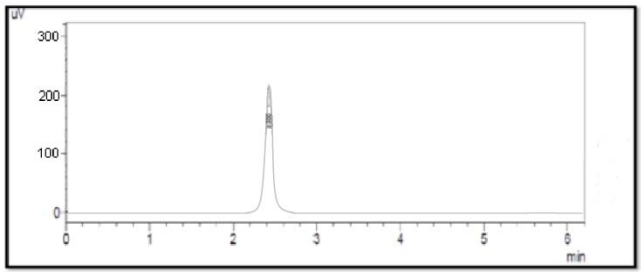

Fig. 4: Chromatogram of double blank (Urine Matrix+ISTD)

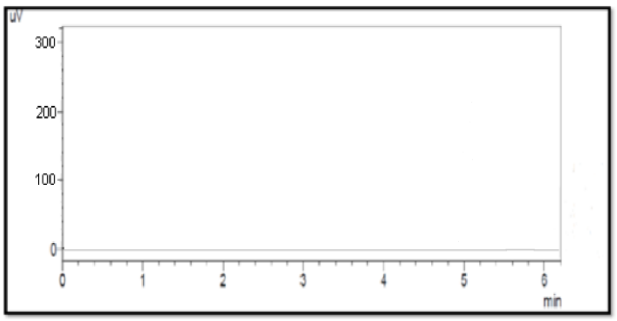

Fig. 3: Chromatogram of blank (Urine Matrix)

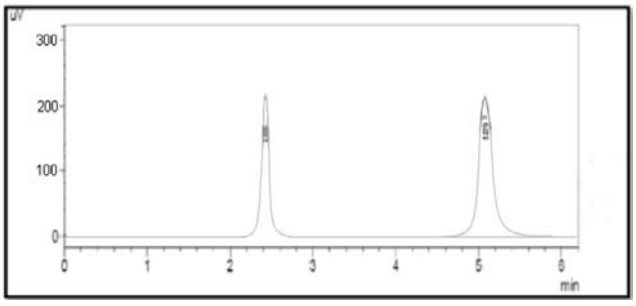

Fig. 5: Chromatogram of standard (Urine Matrix+ISTD+Drug)

Table 2: Acceptance criteria of system suitability

Parameter	Acceptance criteria	HCG	ISTD
Retention time	<2 %CV	5.279±0.00942	2.343±0.00170
Tailing factor	<1.5	1.151±0.000816	1.224±0.00106
Resolution	>2	13.353±0.0593	0.00±0.00
Theoretical plate	>2000	3261±28.005	2160.5±20.814

Table 3: Specificity of HCG

S. No. n=6	Urine sample	Interference at retention time of drugs		Interference at retention time of ISTD	
		Retention time (min)	Peak area	Retention time	Peak area
1	A	Not applicable (NA)	Not detected (ND)	Not applicable (NA)	Not detected (ND)
2	B				
3	C				
4	D				
5	E				
6	F				

Table 4: Specificity of HCG (Extracted LLOQ sample)

Extracted LLOQ samples				
Sample No. n=6	HCG		Internal standard	
	RT (min)	Peak Area	RT (min)	Peak Area
1	5.279	26589	2.342	38779
2	5.278	26541	2.342	38845
3	5.278	26540	2.341	38782
4	5.279	26579	2.342	38989
5	5.279	26603	2.344	38752
6	5.278	26591	2.343	39193
Mean	5.278	26573.83	2.342	38890
SD	0.0005	24.5792	0.00094	18.2452
%CV	9.47%	0.0924%	0.040%	0.046%

Linearity and range

Various concentrations from standard HCG, working solution of HCG and ISTD were prepared and calibration graph was plotted between the peak area and concentration (µg/ml). It was found to be linear over the concentration range of 0.37µg/ml to 48.4 µg/ml with R^2value 0.9983. Eight concentrations of solutions were prepared according to the C_{max} value of the drug. The result of linearity and range is shown in the table: 5.

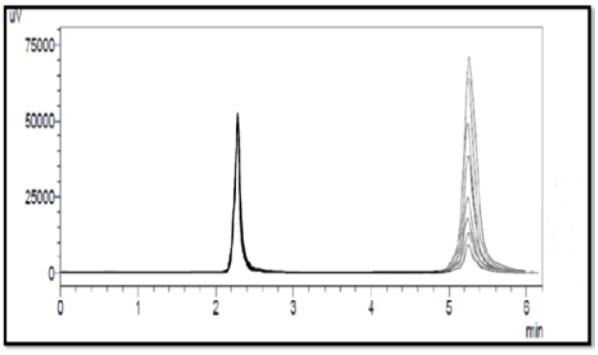

Fig. 6: Overlay chromatogram of standard solution

Accuracy

It is defined as the closeness of mean test results obtained by the method to the actual value (concentration) of the analyte. The accuracy of the method was established using recovery technique (ISTD was also added). Minimum five determinations per concentrations were done to determine LQC, MQC and HQC. Accuracy for HCG was found to be 97.20% to 101.29%, which lies within the acceptance range of±15% and±20% for LQC, MQC and HQC respectively. The result of % accuracy and % CV is shown in table: 6 and table: 7.

Precision

It is defined as the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of single homogenous volume of biological matrix. The precision of the developed method was studied by interday precision and intraday precision method. Minimum five determinations per concentrations were done to determine LQC, MQC and HQC. Repeatability of the method was checked by injecting each concentration solution for five times on the same day as intraday precision and injecting each concentration solution for five times on the different day as interday precision.

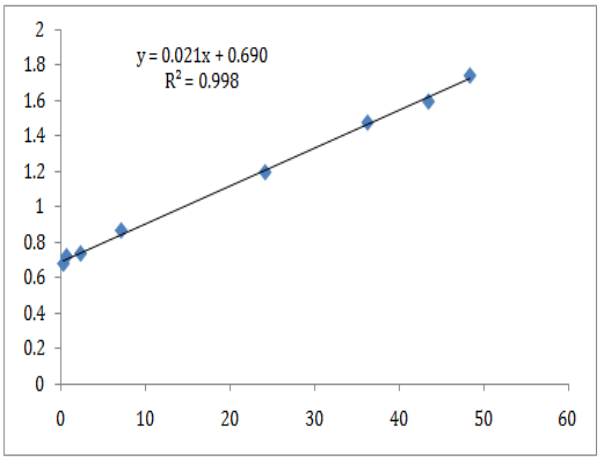

Fig. 6: Calibration curve for HCG

Table 5: Linearity data of HCG

S. No.	Concentration of HCG	Area of HCG	Area of internal standard	Area of HCG/Area of I. S.
1	0.37	26589	39184	0.6785
2	0.74	27944	38810	0.7200
3	2.4	28872	39221	0.7361
4	7.2	33877	39120	0.8659
5	24.2	46780	39157	1.1946
6	36.3	57205	38779	1.4751
7	43.5	62480	39206	1.5936
8	48.4	67859	38998	1.7400

Table 6: Accuracy for HCG

QC Samples	Conc. (µg/ml) n=5	Area ratio= Area of HCG / Area of ISTD					Mean	%Accuracy
		Set1	Set 2	Set 3	Set 4	Set 5		
LQC	1.11	0.7102	0.7137	0.7140	0.7128	0.7181	0.7137	97.20%
MQC	24.2	1.1946	1.2042	1.1950	1.1974	1.2039	1.1990	97.80%
HQC	36.3	1.4599	1.4703	1.4702	1.4611	1.4785	1.468	101.29%

Table 7: % CV obtained for accuracy of HCG

	LQC	MQC	HQC
Concentration	1.11	24.2	36.3
Mean	0.7137	1.1990	1.468
SD	0.002549	0.004218	0.006835
% CV	0.3571%	0.3517%	0.4655%

Table 8: Intraday precision for HCG

QC Samples	Conc. (µg/ml) n=5	Area ratio= Area of HCG / Area of ISTD					Mean	SD	%CV
		Set1	Set 2	Set 3	Set 4	Set 5			
LQC	1.11	0.7201	0.7141	0.7184	0.7166	0.7193	0.7277	0.00214	0.2940%
MQC	24.2	1.1966	1.2023	1.1943	1.1982	1.2031	1.1989	0.00335	0.2794%
HQC	36.3	1.4740	1.4725	1.4728	1.4656	1.4605	1.4690	0.00307	0.2089%

Table 9: Interday precision for HCG (Day 1)

Day 1									
QC Samples	Conc. (µg/ml) n=5	Area ratio= Area of HCG / Area of ISTD					Mean	SD	%CV
		Set1	Set 2	Set 3	Set 4	Set 5			
LQC	1.11	0.7201	0.7141	0.7184	0.7166	0.7193	0.7277	0.00214	0.2940%
MQC	24.2	1.1966	1.2023	1.1943	1.1982	1.2031	1.1989	0.00335	0.2794%
HQC	36.3	1.4740	1.4725	1.4728	1.4656	1.4605	1.4690	0.00307	0.2089%

Table 10: Interday precision for HCG (Day 2)

Day 2									
QC Samples	Conc. (µg/ml) n=5	Area ratio= Area of HCG / Area of ISTD					Mean	SD	%CV
		Set1	Set 2	Set 3	Set 4	Set 5			
LQC	1.11	0.7196	0.7193	0.7180	0.7148	0.7190	0.7191	0.00175	0.2433%
MQC	24.2	1.2000	1.1958	1.1979	1.1959	1.1992	1.1977	0.00169	0.1411%
HQC	36.3	1.4617	1.4731	1.4744	1.4704	1.4696	1.4698	0.00442	0.3007%

Table 11: Interday precision for HCG (Day 3)

Day 3									
QC Samples	Conc. (µg/ml) n=5	Area ratio= Area of HCG / Area of ISTD					Mean	SD	%CV
		Set1	Set 2	Set 3	Set 4	Set 5			
LQC	1.11	0.7195	0.7190	0.7182	0.7140	0.7187	0.7178	0.00198	0.2758%
MQC	24.2	1.1947	1.2038	1.2000	1.1968	1.1964	1.1983	0.00322	0.2687%
HQC	36.3	1.4592	1.4591	1.4646	1.4716	1.4691	1.4647	0.00507	0.3461%

Intraday precision for HCG

% CV of intraday precision for MQC and HQC were found to be 0.1411 to 0.3461%, which lies within the acceptance range of±15%. % CV for LQC sample was found to be 0.2433% to 0.2940%, which lies within the acceptance range of±20%. % CV of intraday precision is shown in the table: 8.

Interday precision for HCG

% CV of intraday precision for MQC and HQC were found to be 0.1411 to 0.3461%, which lies within the acceptance range of±15% and % CV for LQC sample was found to be 0.2433% to 0.2940%, which lies within the acceptance range of±20%. The % CV of intraday precision is shown in the table: 9, table: 10 and table: 11.

Recovery

It is defined as the detector response obtained from an amount of analyte added and extracted from the biological matrix. Recovery should not be 100%. Repeatability was checked by injecting each concentration of six determinations such as LQC, MQC and HQC respectively. % recovery of HCG for LQC, MQC and HQC was found to be 94.98%, 95.88% and 95.21% respectively which is in the acceptance limit less than 100%. The result of recovery is shown in the table: 12, table: 13 and table: 14.

Table 12: % Recovery for HCG (LQC)

Sample No. n=6	LQC		
	Human Chorionic Gonadotropin (HCG)		
	Area ratio of extracted	Area ratio of post extracted	% Recovery
1	0.7191	0.7513	95.71%
2	0.7140	0.7491	95.31%
3	0.7169	0.7583	94.54%
4	0.7132	0.7545	94.52%
5	0.7142	0.7515	95.03%
6	0.7137	0.7530	94.78%
Mean	0.7151	0.7529	94.98%
SD	0.00211	0.00290	0.4261
%CV	0.2950%	0.3851%	0.4486%

Table 13: % Recovery for HCG (MQC)

Sample No. n=6	MQC		
	Human Chorionic Gonadotropin (HCG)		
	Area ratio of extracted	Area ratio post extracted	% Recovery
1	1.1995	1.2578	95.36%
2	1.1943	1.2578	94.95%
3	1.2039	1.2319	97.72%
4	1.1926	1.2520	95.25%
5	1.1945	1.2477	95.73%
6	1.2013	1.2475	96.29%
Mean	1.1976	1.2491	95.88%
SD	0.00413	0.00875	0.9227
%CV	0.3448%	0.7005	0.9623%

Table 14: % Recovery for HCG (HQC)

Sample No. n=6	HQC		
	Human Chorionic Gonadotropin (HCG)		
	Area ratio of extracted	Area ratio post extracted	% Recovery
1	1.4614	1.5244	95.86%
2	1.4616	1.5249	95.84%
3	1.4609	1.5250	95.79%
4	1.4661	1.5324	95.37%
5	1.4620	1.5265	95.77%
6	1.4688	1.5255	96.28%
Mean	1.4684	1.5264	95.21%
SD	0.00294	0.00273	0.2343
%CV	0.2002%	0.1788%	0.2460%

Stability study

Different stability studies for HCG including short term stability, freeze thaw stability, long term stability and stock solution stability were performed as per USFDA guideline and observed data are given in table: 15 to table: 19. % CV is observed within acceptance criteria.

Short term stability study

Stability was done in biological sample and aliquot of low and high should be kept for minimum 7 hours at room temperature.

Long term stability study

The stability study was done for the time between 0 to 10 days.

Freeze-thaw solution stability

The stability study was done for minimum three freeze thaw cycle. Spiked sample at LQC and HQC levels were stored in deep freezer for at least 24 hrs for FT cycle 1. Then thaw at room temperature. This process was again repeated for 2 to 3 freeze-thaw cycle and then compare with fresh sample.

Stock solution stability

The stability study was done for the time between 0 to 6 hr at room temperature and the time between 0 to 10 days at refrigerated condition (2-8 °C) for the drug and internal standard.

Table 15: Short term stability for HCG

QC Samples	LQC		HQC	
S. No.	Area ratio of HCG		Area ratio of HCG	
n=5	Fresh	After 7 hr	Fresh	After 7 hr
1	0.7200	0.7194	1.4624	1.4628
2	0.7197	0.7185	1.4640	1.4626
3	0.7194	0.7181	1.4630	1.4633
4	0.7194	0.7196	1.4615	1.4627
5	0.7193	0.7187	1.4617	1.4640
Mean	0.7195	0.7188	1.4625	1.4640
SD	0.00025	0.00056	0.00091	0.00051
%CV	0.0347%	0.0779%	0.0622%	0.0348%

Table 16: Long term stability for HCG

QC Samples	LQC		HQC	
S. No.	Area ratio of HCG		Area ratio of HCG	
n=5	Fresh	After 10 days	Fresh	After 10 days
1	0.7202	0.7199	1.4616	1.4616
2	0.7202	0.7196	1.4615	1.4618
3	0.7191	0.7203	1.4619	1.4620
4	0.7191	0.7200	1.4608	1.4609
5	0.7195	0.7185	1.4606	1.4607
Mean	0.7196	0.7196	1.4612	1.4614
SD	0.00049	0.00028	0.00049	0.00050
%CV	0.0680%	0.0389%	0.0335%	0.0342%

Table 17: Freeze Thaw stability for HCG

QC Samples	LQC		HQC	
S. No.	Area ratio of HCG		Area ratio of HCG	
n=5	Fresh	After 3 cycles	Fresh	After 3 cycles
1	0.7193	0.7192	1.4632	1.4628
2	0.7196	0.7189	1.4620	1.4619
3	0.7208	0.7204	1.4612	1.4619
4	0.7209	0.7200	1.4608	1.4617
5	0.7206	0.7192	1.4615	1.4620
Mean	0.7202	0.7195	1.4617	1.4620
SD	0.00065	0.00064	0.00082	0.00038
%CV	0.0902%	0.0889%	0.0560%	0.0259%

Stock solution stability at room temperature

Table 18: Stock solution stability for HCG at room Temperature after 6 hrs

QC Samples	LQC		HQC	
S. No.	Area ratio of HCG		Area ratio of HCG	
n=5	Fresh	After 6 hr at RT	Fresh	After 6 hr at RT
1	0.7188	0.7181	1.4629	1.4628
2	0.7202	0.7197	1.4625	1.4625
3	0.7199	0.7201	1.4632	1.4628
4	0.7198	0.7199	1.4637	1.4631
5	0.7190	0.7202	1.4630	1.4629
Mean	0.7195	0.7196	1.4630	1.4628
SD	0.00054	0.000769	0.000392	0.000193
%CV	0.0750%	0.1068%	0.0267%	0.0131%

Stock solution stability at Refrigerated Condition (2-8˚C) for 10 days

Table 19: Stock solution stability for HCG at Refrigerated Condition (2-8˚C) for 10 days

QC Samples	LQC		HQC	
S. No. n=5	Area ratio of HCG		Area ratio of HCG	
	Fresh	After 10 days at 2-8 ˚C	Fresh	After 10 days at 2-8 ˚C
1	0.7198	0.7195	1.4631	1.4621
2	0.7197	0.7190	1.4630	1.4635
3	0.7192	0.7184	1.4614	1.4621
4	0.7192	0.7191	1.4634	1.4640
5	0.7194	0.7197	1.4637	1.4637
Mean	0.7194	0.7191	1.4692	1.4630
SD	0.000249	0.000449	0.000798	0.000815
%CV	0.0346%	0.0624%	0.0543%	0.0557%

CONCLUSION

In present work, human chorionic gonadotropin (HCG) was estimated in human urine by RP-HPLC method. The developed method is highly accurate, precise and specific. It is validated as per USFDA guideline. The method was developed in the mobile phase sodium phosphate buffer (pH 7.0, 0.05M): acetonitrile (87.5:12.5 % v/v) with flow rate 1 ml/min. at 215 nm. Bovine serum albumin (BSA) was used as an internal standard. Very high extraction efficiency (more than 94%) and LLOQ were found to be 0.37 µg/ml. Calibration curve was linear within the range of 0.37 to 48.4 µg/ml. R^2 value was found to be 0.9983 and accuracy was found for LQC, MQC and HQC in between 97.20% to 101.29%. Precision for HCG was found for LQC, MQC and HQC was 0.14 to 0.367 %CV respectively. Stability study like short term, freeze and thaw, long term, and stock solution stability were performed and drug was found to be stable, throughout the study. Thus, this method can be useful for determination of HCG in biological samples in clinical and biomedical research.

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CONFLICT OF INTERESTS

Declared None

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