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A VALIDATION STUDY OF THE BILE ACID DEVELOPED METHOD USING POST-COLUMN DERIVATIZATION IN HPLC

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ABSTRACT:

Bile acid, derived from cholinergic acid (steroidal parent nucleus), aids digestion, absorption, and metabolism. Bile acids govern self-metabolism, lipid metabolism, energy balance, and glucose metabolism. Research on bile acid variations due to metabolism, illness, or individual differences is hot. Bile acid detection uses enzyme analysis, immunoassays, and chromatography. Clinical and laboratory research use these methodologies to varied degrees. Mainstream detection technology is continually upgraded and changed, delivering new detection methods. Gas chromatography (GS) and GC-MS were the most prevalent methods for bile acid testing. HPLC-MS/MS has quickly progressed and became the standard bile acid sample separation and detection method in recent years. This validation study discusses HPLC detection approaches by estimating bile acid using 24 phenacyl ester [derivaizing agent) in post column derivatization technique.

Keywords: Estimation, Bile Acid , Post Column Derivatization and HPLC.

INTRODUCTION:

Derivatization changes a chemical or treatment's molecular structure. Molecular reformations may be needed. This makes extracting, separating, and identifying components easier. Pharmaceuticals can improve their UV-visible detection, fluorescence and chemiluminescence detection, electrochemical detection, and mass spectroscopic detection capabilities by synthesizing cyclic derivatives and separating enantiomers. Derivatization may also improve the medicine's selectivity, sensitivity, thermal stability, and volatility. Derivatization methods are widely used for functional groupings. These functional categories include acids and bases with limited chromophore sites or UV detection. Bile, fatty, amino, and gossypol stereoisomers are further examples (1-3).

It also works for less sensitive and selective metabolites, phytoconstituents, medicines, and

insecticides. The derivatization method must follow all practical approach criteria, especially those related to repeatability and recovery. Pre- or post-column derivatization may occur in high-performance liquid chromatography (HPLC). HPLC analysis also uses Postcolumn derivatization. Chemical derivatization improves analytes chromatographic and mass spectrometric characteristics. Derivatization is commonly needed for gas chromatography (GC) volatility (4).

Selectively derivatizing analytes utilizing reversed-phase (RP) separation or ion-exchange processes after adding a nonpolar group or charged function reduces sample interferences in liquid chromatography. This improves analyte separation. These methods decrease sample (LC) interferences. When investigating molecules with permanent charges, such as the positive charge of a quaternary ammonium component (MS), electrospray ionization (ESI) mass spectrometry may provide more accurate findings. Derivatization usually predicts tandem MS (MS/MS) fragmentation events and increases technique specificity. Capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC) are employed most often to identify chemical molecules in complex matrices. Due to their structural incompatibility with standard HPLC or CE detectors, many compounds of interest have not been discovered. Many chemicals exhibit this. Fluorescence and UV light absorption are examples. Derivatizing the compounds to add chromophoric or fluorophore groups may address the problem. It'll work (5, 6).

LITERATURE REVIEW:

Bile acids are essential for digestion, intestinal microbiota management, and the regulation of glucose, lipid, and cholesterol homeostasis-required pathways. Consequently, it is essential to measure bile acid levels in both healthy and sick individuals. In cirrhosis, the liver, which is responsible for bile acid synthesis, is obliterated, and the progression of the disease is influenced by altered intestinal microbiota, which may also impact bile acid metabolism (7). Several methods have been outlined for the analysis of bile acids in biological fluids such as bile, plasma, urine, and excretion. Given the variety of bile acids found in defecation and their wide spectrum of polarity, analyzing bile acids from faeces is the most difficult operation. Customarily, GC, HPLC, and

their combination with MS are utilised for the measurement of specific bile acids (8). Currently, HPLC-MS is the most technologically advanced of these analytical techniques. Bile acid profiles can be evaluated without the need for time-consuming sample purification beforehand. Several published works have made use of this methodology. Previous research has not adequately documented the extraction of bile acids from faeces, despite these advantages. Moreover, the substantial administrative costs associated with such technologies prevent their widespread adoption. Therefore, it would be advantageous to develop a rapid and dependable HPLC method for faecal bile acids (9, 10).

MATERIAL AND METHOD:

Linearity:

The linearity of an analytical method is its capacity to generate test results that are proportional to the concentration of an analyte in a sample, either directly or through a well-defined mathematical transformation (11-13). The linearity analysis must be performed at six levels across the range of LOQ to 200% for Bile acids at the standard concentration and known impurities at the specification limit. Prepare a series of linearity solutions by diluting the mix impurity solution (Solution H) and the Cholic acid Standard solution (Solution J) to obtain concentrations in the range of LOQ to 200% of known impurities at specification limit and Cholic acid at standard concentration (i.e. LOQ, 50%, 75%, 100%, 150%, and 200%). Prepare each level's linearity study solutions in triplicate and inject a single sample at each level.

1. **Preparation of Solutions:**

   Prepare the linearity standard solution by making serial dilutions of Solution H and Solution J as per given in table (a).

Accuracy:

The degree to which test findings from an analytical technique match the real value indicates the method's accuracy. A common way to represent accuracy is as a percentage of the applied known amount of analyte. Accuracy may be thought of as a measurement of how precise an analytical approach is. Perform accuracy at four levels, namely LOQ, 50%, 100%, and 200%, over the range of known impurities from LOQ to 200% at the specification limit (14). Calculating the % recovery at each level after spitting a predetermined amount of known contaminants into the sample solution. Prepare three control sample

solutions in accordance with the protocol, then determine the impurity percentage. If a known impurity is detected, the recovery must be computed after deducting the impurity from each accuracy level. At each level, prepare the accuracy study samples in duplicate, and then inject only one sample (15).

1. **Preparation of Accuracy Sample Solution:**

Add respective mL of solution A, solution B, solution C, solution D, solution E, solution F and solution G as per given in table (b).

Precision:

The degree of agreement between series of measurements acquired from numerous samplings of the same homogenous sample under the specified conditions defines an analytical method's precision (16).

1. **System Precision:**

By injecting the sensitivity solution, the system suitability solution, and six copies of the standard solution from the same HPLC vial in accordance with the test technique, you may determine the system precision (17).

2. **Method Precision (Repeatability):**

Repeatability described the accuracy over a brief period of time when working under the same conditions (18).

3. **Intermediate Precision:**

On a separate day, by a different analyst, using a different HPLC machine, using a different column of the same brand, and using the same batch of samples as described under repeatability, intermediate precision indicated within the laboratory variance (19).

RESULT AND DISCUSSION:

Linearity:

A linearity analysis was conducted at six levels spanning the range of LOQ to 200% for Cholic acid at the standard concentration and known impurities at the specification limit. Prepared a series of linearity solutions by quantitatively diluting the Mix impurity solution (Solution H) and the Cholic acid standard solution (Solution J) to obtain concentrations in the range of LOQ to 200% of known impurities at specification limit at standard concentration (i.e. LOQ, 50%, 75%, 100%, 150% and 200%) (Tables c, d, e, f, g, and h).

- Acceptance Criteria:

The correlation coefficient ('R') value should not be less than 0.99 over the working range.

- Correlation coefficient ('R') value (Figure a, b, c, d, e, f, ad g)

  For Taurocholic acid = 0.999970

  For Cholic acid = 0.999863

  For Taurochenodeoxycholic acid = 0.999980

  For Glycocholic acid = 0.999986

  For Glycochenodeoxycholic acid = 0.999028

  For Deoxycholic acid = 0.999981

  For Chenodeoxycholic acid= 0.999981

Accuracy:

Accuracy is frequently expressed as a percentage of the known quantity of analyte applied. Over the range of LOQ to 200% of known impurities at specification limit, four levels of precision were evaluated: LOQ, 50%, 100%, and 200%. Spiking a known quantity of known impurities into the sample solution and calculating the percent recovery at each level (Tables i, j, k, l, m, n, and o).

- **Acceptance Criteria:**

  i) The recovery of known impurities at the LOQ Level should be between 70 and 130 percent.

  ii) Recovery and overall mean recovery of known impurities at each level (except LOQ Level) must fall between 80 and 120 percent.

  iii) The overall % RSD for the recovery of known impurities (excluding the LOQ level) must not exceed 5.0.

- **Observation:**

  1. The recovery of known impurities at the LOQ level ranged from 70.0% to 130.0%.
  2. The recovery and overall mean recovery of known impurities at each level ranged from 80 to 120 percent.
  3. The overall percent RSD for the recovery of known impurities is less than 5.0

- **Conclusion:**

  All results are well within the criteria for acceptance. Therefore, the technique is valid. Accuracy Graph of Taurocholic acid quantity added versus quantity detected.

Precision:

Precision of an analytical method is the degree of agreement between series of measurements acquired from numerous samples of the same homogeneous sample under the specified conditions.

1. **System Precision:**

   By injecting six duplicates of the standard solution from the same HPLC vial alongside the sensitivity

solution, system suitability solution, and system precision solution in accordance with the protocol. The data set's table (p) contains the results.

- **Acceptance Criteria:**

  i) Signal-to-noise ratio: The signal-to-noise ratio for the Cholic acid peak in the sensitivity solution must be greater than 10.

  ii) Resolution: The resolution between the Cholic acid and Taurocholic acid peaks in the system suitability solution must not be less than 1.5.

  iii) Tailing factor: The tailing factor of the Cholic acid peak derived from the initial injection of the standard solution must not exceed 2.0.

  iv) Theoretical plates: Theoretical plates of the Cholic acid peak derived from the initial injection of the standard solution must not be less than 2000.

  v) RSD: The relative standard deviation of the Cholic acid peak area derived from six replicate administrations of standard solution must not exceed 5%.

- **Observation:**

  S/N Ratio = 175

  Resolution = 10.2

  Tailing factor = 1.0

  Theoretical plates = 517704

  % RSD = 4.9

- **Conclusion:**

All results are well within the criteria for acceptance. Therefore, the system is accurate.

2. Method Precision (Repeatability):

Repeatability referred to the precision over a limited period of time when operating under identical conditions. Using the same set of samples, three control sample solutions and six sample contaminated solutions (known impurity at specified level) were prepared. As a control for the calculation of associated chemicals, three control sample solutions were utilised. Determined the percentage RSD of known contaminants in six spiking sample solutions. Tables (q) and (r) in the report detail the results.

- **Acceptance Criteria:**

RSD for percent by weight of known impurities in six contaminated sample solutions must not exceed 10%.

- **Observation:**

  RSD for % w/w of known impurities in six spiked sample solution,

  Taurocholic acid = 0.0%

Cholic acid = 0.0%

Taurochenodeoxycholic acid = 0.0%

Glycocholic acid = 0.0%

Glycochenodeoxycholic acid = 0.0%

Deoxycholic acid = 2.7%

Chenodeoxycholic acid = 2.6%

- **Conclusion:**

All results are found well within the acceptance criteria. Hence method is precise.

3. Intermediate Precision:

As described under repeatability, on a separate day, by a different analyst, utilizing a different HPLC machine, utilizing a different column of the same brand, and using the same batch of samples, intermediate precision was represented within the laboratory variance. Three control sample solutions and six sample spiked solutions (spiking of known impurity at specification level) were prepared on a different day, by a different analyst, using a different HPLC system, a different column of the same make, and the same lot of samples from each Bile acid sample. In six spiking sample solutions, the percent RSD of percent w/w of known contaminants was determined (tables s, t). Using procedure precision and intermediate precision, it was determined that the overall% RSD of known contaminants in twelve spiking sample solutions was% w/w (table u).

- **Acceptance Criteria:**

i) The RSD for percent weight-to-weight of known impurities in six injected sample solutions should not exceed 10%.

ii) The total RSD for % w/w of known impurities in twelve spiked sample solutions derived from method precision and intermediate precision must not exceed 15%. All results are found well within the acceptance criteria. Hence method is precise and rugged.

Figures and Tables:

Figures:

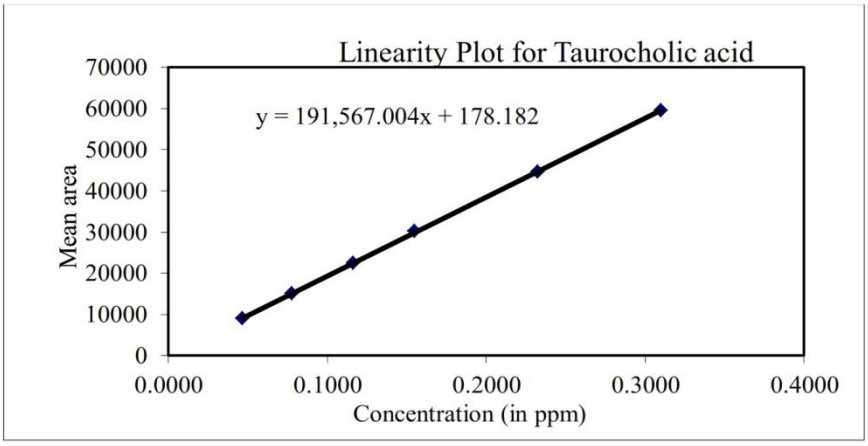

Figure (a) Linearity Plot for Taurocholic acid for bile acids (Postcolumn Derivatization)

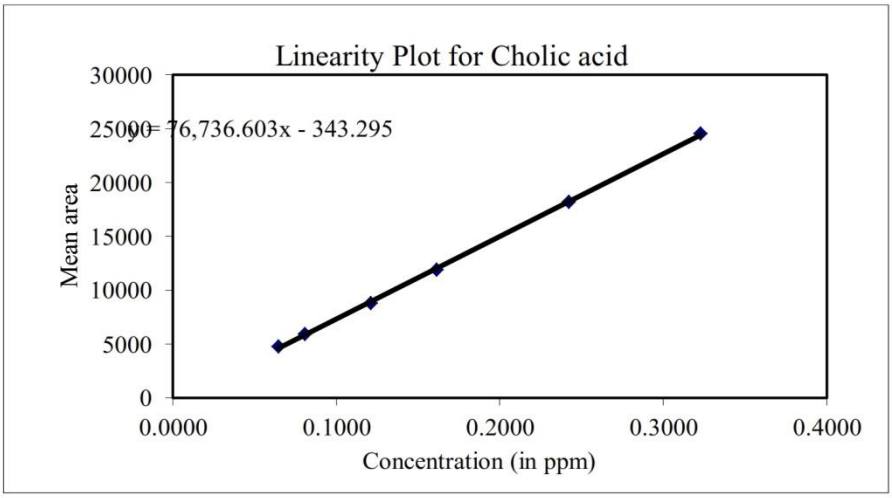

Figure (b) Linearity Plot for Cholic acid for bile acids (Postcolumn Derivatization)

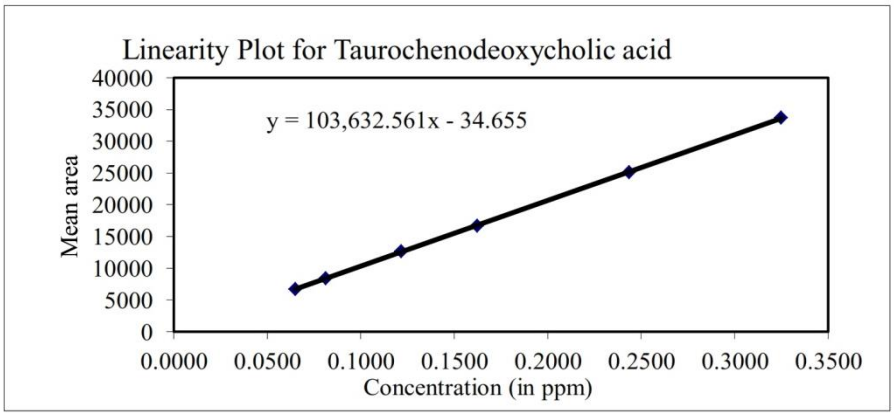

Figure (c) Linearity Plot for Taurochenodeoxycholic acid for bile acids (Postcolumn Derivatization)

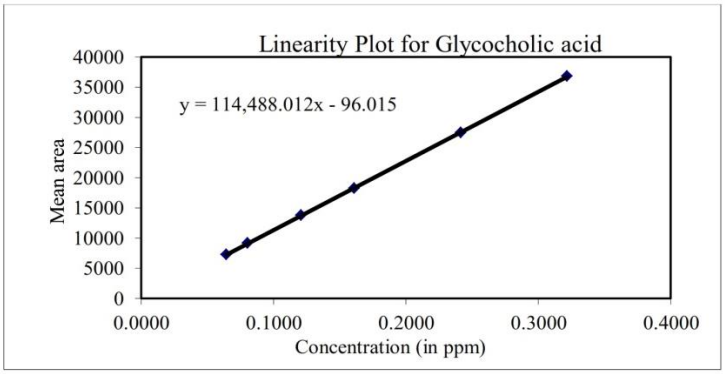

Figure (d) Linearity Plot for Glycocholic acid for bile acids (Postcolumn Derivatization)

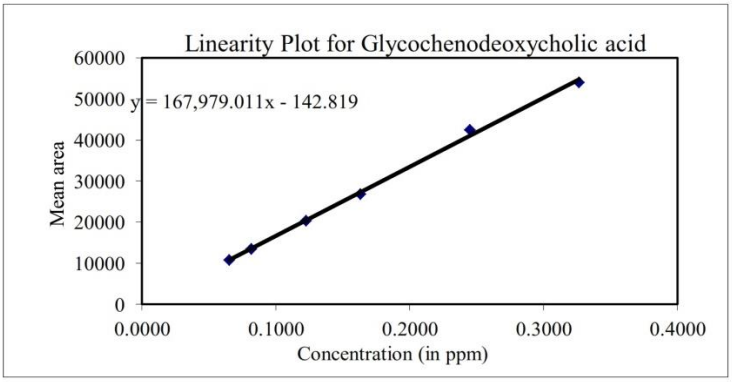

Figure (e) Linearity Plot for Glycochenodeoxycholic acid for bile acids (Postcolumn Derivatization)

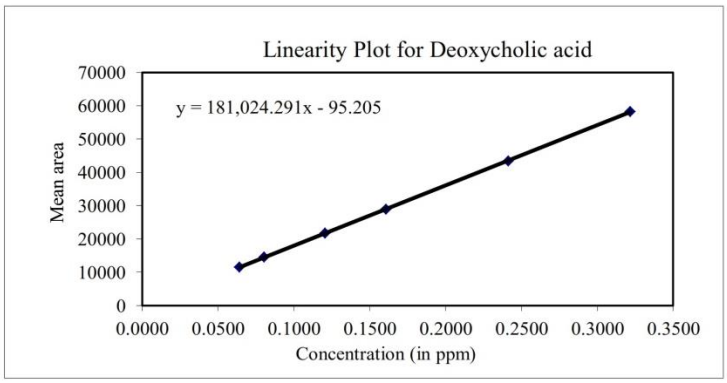

Figure (f) Linearity Plot for Deoxycholic acid for bile acids (Postcolumn Derivatization)

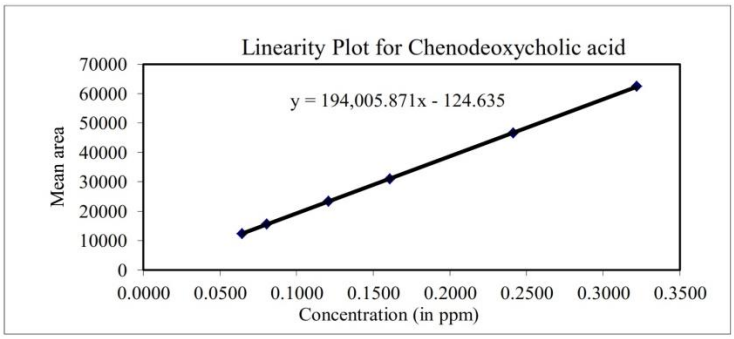

Figure (g) Linearity Plot for Chenodeoxycholic acid for bile acids (Postcolumn Derivatization

Tables:

Table (a) Preparation of Linearity standard solutions

Sr. No	Conc. of known impurities w.r.t. specification limit and Cholic acid w.r.t. standard conc. (in %)	Added Solutions (in mL)		Dilution (in mL)	Conc. of known impurities (in ppm)	Conc. of Bile acid (in ppm)
		Solution H	Solution J			
1	LOQ	0.8	0.6	100	0.064	0.064
2	50	1.0	1.0	100	0.08	0.08
3	75	1.5	1.5	100	0.12	0.12
4	100	2.0	2.0	100	0.16	0.16
5	150	3.0	3.0	100	0.24	0.24
6	200	4.0	4.0	100	0.32	0.32

Table (b) Preparation of accuracy sample solutions

Sr. No.	% Conc. of known impurities w.r.t. specification limit (in %)	Solution added (in mL)							Dilution (in mL)	2nd Dilution		Conc. of Known impurities (in ppm)
		A	B	C	D	E	F	G		mL	mL	
1	LOQ	2.0	2.0	2.0	2.0	2.0	2.0	2.0	1000	8	100	0.064
2	50	2.5	2.5	2.5	2.5	2.5	2.5	2.5	1000	8	100	0.08
3	100	5.0	5.0	5.0	5.0	5.0	5.0	5.0	1000	8	100	0.16
4	200	7.5	7.5	7.5	7.5	7.5	7.5	7.5	1000	8	100	0.32

Table (c) Linearity study data of Cholic acid for bile acids (Postcolumn Derivatization)

Level	Conc. of Cholic acid w.r.t. specification limit (%)	Concentration (ppm)	Replicates	Peak Area	Mean Peak Area
1	LOQ	0.0646	1	4827	4753
			2	4646	
			3	4787	
2	50	0.0807	1	5858	5932
			2	6008	
			3	5930	
3	75	0.1211	1	8801	8790
			2	8860	
			3	8708	
4	100	0.1614	1	11898	11917
			2	11958	
			3	11894	
5	150	0.2422	1	18134	18198
			2	18280	
			3	18180	
6	200	0.3229	1	24620	24542
			2	24281	
			3	24726	
Correlation coefficient (R)					0.999863
Y intercept					-343.295
% Y intercept					-2.881
Slope					76736.603
Residual sum of square					80671.011

Table (d) Linearity study data of Taurochenodeoxycholic acid for bile acids (Postcolumn Derivatization)

Level	Conc. of Taurochenodeoxycholic acid w.r.t. specification limit (%)	Concentration (ppm)	Replicates	Peak Area	Mean Peak Area
1	LOQ	0.0650	1	6646	6686
			2	6739	
			3	6672	
2	50	0.0812	1	8328	8416
			2	8586	
			3	8334	
3	75	0.1218	1	12633	12652
			2	12652	
			3	12672	
4	100	0.1624	1	16719	16729
			2	16736	
			3	16731	
5	150	0.2437	1	25144	25135
			2	25162	
			3	25098	
6	200	0.3249	1	33727	33703
			2	33674	
			3	33707	
Correlation coefficient (R)					0.999980
Y intercept					-34.655
% Y intercept					-0.207
Slope					103632.561
Residual sum of square					21902.795

Table (e) Linearity study data of Glycocholic acid for bile acids (Postcolumn Derivatization)

Level	Conc. of Glycocholic acidw.r.t. specification limit (%)	Concentration (ppm)	Replicates	Peak Area	Mean Peak Area
1	LOQ	0.0643	1	7239	7258
			2	7262	
			3	7273	
2	50	0.0804	1	9106	9153
			2	9228	
			3	9126	
3	75	0.1206	1	13705	13744
			2	13786	
			3	13741	
4	100	0.1608	1	18277	18261
			2	18241	
			3	18264	
5	150	0.2413	1	27372	27445
			2	27468	
			3	27495	
6	200	0.3217	1	36811	36803
			2	36834	
			3	36764	
Correlation coefficient (R)					0.999986
Y intercept					-96.015
% Y intercept					-0.526
Slope					114488.012
Residual sum of square					17724.420

Table (f) Linearity study data of Glycochenodeoxycholic acid for bile acids (Postcolumn Derivatization)

Level	Conc. of Glycochenodeoxycholic acid analog w.r.t. specification limit (%)	Concentration (ppm)	Replicates	Peak Area	Mean Peak Area
1	LOQ	0.0653	1	10774	10764
			2	10789	
			3	10729	
2	50	0.0817	1	13418	13526
			2	13756	
			3	13403	
3	75	0.1225	1	20247	20370
			2	20476	
			3	20388	
4	100	0.1633	1	26852	26846
			2	26852	
			3	26833	
5	150	0.2450	1	40235	42422
			2	43412	
			3	43618	
6	200	0.3267	1	53945	53950
			2	53995	
			3	53910	
Correlation coefficient (R)					0.999028
Y intercept					-142.819
Slope					167979.011
Residual sum of square					2812243.817

Table (g) Linearity study data of Deoxycholic acid for bile acids (Postcolumn Derivatization)

Level	Conc. of Deoxycholic acid w.r.t. specification limit (%)	Concentration (ppm)	Replicates	Peak Area	Mean Peak Area
1	LOQ	0.0643	1	11418	11501
			2	11489	
			3	11597	
2	50	0.0804	1	14427	14561
			2	14789	
			3	14466	
3	75	0.1206	1	21780	21786
			2	21901	
			3	21676	
4	100	0.1609	1	28991	28970
			2	29011	
			3	28908	
5	150	0.2413	1	43228	43419
			2	43412	
			3	43618	
6	200	0.3217	1	58116	58261
			2	58457	
			3	58209	
Correlation coefficient (R)					0.999981
Y intercept					-95.205
% Y intercept					-0.329
Slope					181024.291
Residual sum of square					60983.057

Table (i) Recovery study data of Taurocholic acid for bile acids (Postcolumn Derivatization)

Level	% Conc. of Taurocholic acid w.r.t. specification limit (%)	Taurocholic acid Added (% w/w)	Taurocholic acid recovered (% w/w)	% Recovery	Mean % Recovery	% RSD	Overall % recovery and overall % RSD
1	LOQ	0.0808	0.0916	113.4	114.5	1.6	Overall % recovery (except LOQ level) = 115.6 Overall % RSD (except LOQ level) = 0.9
		0.0808	0.0943	116.7			
		0.0808	0.0917	113.5			
2	50	0.100	0.115	115.0	115.0	0.9	
		0.100	0.114	114.0			
		0.100	0.116	116.0			
3	100	0.199	0.227	114.1	115.4	1.0	
		0.199	0.231	116.1			
		0.199	0.231	116.1			
4	200	0.399	0.461	115.5	116.3	0.7	
		0.399	0.465	116.5			
		0.399	0.467	117.0			

Table (h) Linearity study data of Chenodeoxycholic acid for bile acids (Postcolumn Derivatization)

Level	Conc. of Chenodeoxycholic acid w.r.t. specification limit (%)	Concentration (ppm)	Replicates	Peak Area	Mean Peak Area
1	LOQ	0.0644	1	12228	12306
			2	12342	
			3	12348	
2	50	0.0804	1	15482	15603
			2	15837	
			3	15490	
3	75	0.1207	1	23272	23341
			2	23431	
			3	23321	
4	100	0.1609	1	30955	31018
			2	31044	
			3	31054	
5	150	0.2413	1	46379	46526
			2	46498	
			3	46702	
6	200	0.3218	1	62415	62427
			2	62261	
			3	62605	
Correlation coefficient (R)					0.999981
% Y intercept					-0.402
Slope					194005.871
Residual sum of square					69622.422

Table (j) Recovery study data of Cholic acid for bile acids (Postcolumn Derivatization)

Level	% Conc. of Cholic acid w.r.t. specification limit (in %)	Cholic acid Added (% w/w)	Cholic acid recovered (% w/w)	% Recovery	Mean % Recovery	% RSD	Overall % recovery and overall % RSD
1	LOQ	0.0797	0.0799	100.3	99.0	1.4	Overall % recovery (except LOQ level) = 99.4 Overall % RSD (except LOQ level) = 0.9
		0.0797	0.0791	99.2			
		0.0797	0.0777	97.5			
2	50	0.100	0.101	101.0	99.7	1.5	
		0.100	0.098	98.0			
		0.100	0.100	100.0			
3	100	0.199	0.197	99.0	98.8	0.3	
		0.199	0.196	98.5			
		0.199	0.197	99.0			
4	200	0.399	0.397	99.5	99.8	0.3	
		0.399	0.399	100.0			
		0.399	0.399	100.0			

Table (k) Recovery study data of Taurochenodeoxycholic acid for bile acids (Postcolumn Derivatization)

Level	% Conc. of Taurocholic acid w.r.t. specification limit (in %)	Taurocholic acid Added (% w/w)	Taurocholic acid recovered (% w/w)	% Recovery	Mean % Recovery	% RSD	Overall % recovery and overall % RSD
1	LOQ	0.0782	0.0793	101.4	102.0	0.6	Overall % recovery (except LOQ level) = 99.1 Overall % RSD (except LOQ level) = 0.9
		0.0782	0.0803	102.7			
		0.0782	0.0797	101.9			
2	50	0.098	0.098	100.0	100.0	1.0	
		0.098	0.097	99.0			
		0.098	0.099	101.0			
3	100	0.196	0.193	98.5	98.7	0.3	
		0.196	0.193	98.5			
		0.196	0.194	99.0			
4	200	0.391	0.385	98.5	98.6	0.1	
		0.391	0.386	98.7			
		0.391	0.386	98.7			

Table (l) Recovery study data of Glycocholic acid for bile acids (Postcolumn Derivatization)

Level	% Conc. of Glycocholic acid w.r.t. specification limit (in %)	Glycocholic acid Added (% w/w)	Glycocholic acid recovered (% w/w)	% Recovery	Mean % Recovery	% RSD	Overall % recovery and overall % RSD
1	LOQ	0.0797	0.0776	97.4	98.2	0.7	Overall % recovery (except LOQ level) = 97.9 Overall % RSD (except LOQ level) = 0.3
		0.0797	0.0784	98.4			
		0.0797	0.0787	98.7			
2	50	0.100	0.098	98.0	97.7	0.6	
		0.100	0.098	98.0			
		0.100	0.097	97.0			
3	100	0.199	0.195	98.0	98.0	0.0	
		0.199	0.195	98.0			
		0.199	0.195	98.0			
4	200	0.398	0.389	97.7	97.9	0.2	
		0.398	0.390	98.0			
		0.398	0.390	98.0			

Table (m) Recovery study data of Glycochenodeoxycholic acid for bile acids (Postcolumn Derivatization)

Level	% Conc. of Glycochenodeoxycholic acid w.r.t. specification limit (%)	Glycochenodeoxycholic acid Added (% w/w)	Glycochenodeoxycholic acid recovered (% w/w)	% Recovery	Mean % Recovery	% RSD	Overall % recovery and overall % RSD
1	LOQ	0.0791	0.0778	98.4	100.8	2.0	Overall % recovery (except LOQ level) = 100.3 Overall % RSD (except LOQ level) = 1.1
		0.0791	0.0808	102.1			
		0.0791	0.0805	101.8			
2	50	0.098	0.098	100.0	101.0	1.8	
		0.098	0.098	100.0			
		0.098	0.101	103.1			
3	100	0.196	0.196	100.0	100.0	0.5	
		0.196	0.195	99.5			
		0.196	0.197	100.5			
4	200	0.392	0.391	99.7	99.9	0.5	
		0.392	0.390	99.5			
		0.392	0.394	100.5			

Table (n) Recovery study data of Deoxycholic acid for bile acids (Postcolumn Derivatization)

Level	% Conc. of Deoxycholic acid w.r.t. specification limit (%)	Deoxycholic acid Added (% w/w)	Deoxycholic acid recovered (% w/w)	% Recovery	Mean % Recovery	% RSD	Overall % recovery and overall % RSD
1	LOQ	0.0780	0.0757	97.1	97.2	0.1	Overall % recovery (except LOQ level) = 96.4 Overall % RSD (except LOQ level) = 0.8
		0.0780	0.0758	97.2			
		0.0780	0.0758	97.2			
2	50	0.097	0.094	96.9	97.2	0.6	
		0.097	0.095	97.9			
		0.097	0.094	96.9			
3	100	0.195	0.186	95.4	95.9	0.5	
		0.195	0.187	95.9			
		0.195	0.188	96.4			
4	200	0.390	0.373	95.6	96.0	0.4	
		0.390	0.375	96.2			
		0.390	0.375	96.2			

Table (o) Recovery study data of Chenodeoxycholic acids for bile acids (Postcolumn Derivatization)

Level	% Conc. of Chenodeoxycholic acids w.r.t. specification limit (%)	Chenodeoxycholic acids Added (% w/w)	Chenodeoxycholic acids recovered (% w/w)	% Recovery	Mean % Recovery	% RSD	Overall % recovery and overall % RSD
1	LOQ	0.0771	0.0774	100.4	100.7	0.3	Overall % recovery (except LOQ level) = 100.3 Overall % RSD (except LOQ level) = 0.4
		0.0771	0.0779	101.0			
		0.0771	0.0777	100.8			
2	50	0.096	0.096	100.0	100.3	0.6	
		0.096	0.097	101.0			
		0.096	0.096	100.0			
3	100	0.193	0.194	100.5	100.2	0.3	
		0.193	0.193	100.0			
		0.193	0.193	100.0			
4	200	0.386	0.386	100.0	100.4	0.5	
		0.386	0.387	100.3			
		0.386	0.390	101.0			

Table (p) System Precision data of Cholic acid for bile acids (Postcolumn Derivatization)

Sr. No.	Replicates of standard solution	Cholic acid peak area
1	Standard solution - 1	33296
2	Standard solution - 2	30187
3	Standard solution - 3	29616
4	Standard solution - 4	29557
5	Standard solution – 5	29481
6	Standard solution – 6	29555
Mean		30282
SD		1498.84
% RSD		4.9
Tailing Factor		1.0
Theoretical plates		517704
Resolution between peaks due to Cholic acid and Taurocholic acid from system suitability solution		10.2

Table (q) Method precision data for three control samples of Bile acid solution for bile acids (Postcolumn Derivatization)

Name of the Impurities	Control sample solutions (% w/w)			
	Sample - 1	Sample – 2	Sample - 3	Mean
Taurocholic acid	ND	ND	ND	NA
Cholic acid	ND	BQL	ND	NA
Taurochenodeoxycholic acid	ND	ND	ND	NA
Glycocholic acid	BQL	BQL	BQL	NA
Glycochenodeoxycholic acid	ND	ND	ND	NA
Deoxycholic acid	ND	ND	ND	NA
Chenodeoxycholic acids	ND	ND	ND	NA
Total impurities	NA	NA	NA	NA

BQL: Below Quantitation Limit ND: Not Detected NA: Not Applicable

Table (r) Method precision data for six sample spiked solutions of Bile Acid Sample of Bile acid solution for bile acids (Postcolumn Derivatization)

Name of the Impurities	Spiked sample solutions (% w/w)								
	1	2	3	4	5	6	Mean	SD	% RSD
Taurocholic acid	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.000	0.0
Cholic acid	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.000	0.0
Taurochenodeoxycholic acid	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.000	0.0
Glycocholic acid	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.000	0.0
Glycocheno-deoxycholic acid	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.000	0.0
Deoxycholicacid	0.18	0.18	0.19	0.19	0.19	0.19	0.19	0.005	2.7
Chenodeoxycholic acid	0.20	0.19	0.20	0.20	0.20	0.19	0.20	0.005	2.6

Table (s) Intermediate precision data for three control samples of Bile acid solution of Bile acid solution for bile acids (Postcolumn Derivatization)

Name of the Impurities	Control sample solutions (% w/w)			
	Sample - 1	Sample – 2	Sample - 3	Mean
Taurocholic acid	ND	ND	ND	NA
Cholic acid	ND	BQL	ND	NA
Taurochenodeoxycholic acid	ND	ND	ND	NA
Glycocholic acid	BQL	BQL	ND	NA
Glycochenodeoxycholic acid	ND	ND	ND	NA
Deoxycholic acid	ND	ND	ND	NA
Chenodeoxycholic acid	ND	ND	ND	NA

BQL: Below Quantitation Limit ND: Not Detected NA: Not Applicable

Table (t) Intermediate precision data for six sample spiked solutions of Bile acid sample

Name of the Impurities	Spiked sample solutions (% w/w)								
	1	2	3	4	5	6	Mean	SD	% RSD
Taurocholic acid	0.16	0.17	0.17	0.17	0.15	0.18	0.17	0.010	6.1
Cholic acid	0.20	0.19	0.19	0.19	0.19	0.19	0.19	0.004	2.1
Taurochenodeoxycholic acid	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.000	0.0
Glycocholic acid	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.000	0.0
Glycochenodeoxycholic acid	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.000	0.0
Deoxycholic acid	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.000	0.0

Table (u) Overall RSD for % w/w of known impurities in twelve spiked sample solutions from method precision and intermediate precision of Bile acid sample solution

Sample	Bile acid sample solution % w/w													
	Taurocholic acid		Cholic acid		Taurochenodeoxycholic acid		Glycocholc acid		Glycochoxy cholic acid		Deoxy choc acid		Chenodeoy cholic acid	
	MP	IP	MP	IP	MP	IP	MP	IP	MP	IP	MP	IP	MP	IP
1	0.17	0.16	0.20	0.20	0.19	0.19	0.20	0.19	0.19	0.19	0.18	0.18	0.20	0.19
2	0.17	0.17	0.20	0.19	0.19	0.19	0.20	0.19	0.19	0.19	0.18	0.18	0.19	0.19
3	0.17	0.17	0.20	0.19	0.19	0.19	0.20	0.19	0.19	0.19	0.19	0.18	0.20	0.19
4	0.17	0.17	0.20	0.19	0.19	0.19	0.20	0.19	0.19	0.19	0.19	0.18	0.20	0.19
5	0.17	0.15	0.20	0.19	0.19	0.19	0.20	0.19	0.19	0.19	0.19	0.18	0.20	0.19
6	0.17	0.18	0.20	0.19	0.19	0.19	0.20	0.19	0.19	0.19	0.19	0.18	0.19	0.19
Mean	0.17	0.17	0.20	0.19	0.19	0.19	0.20	0.19	0.19	0.19	0.19	0.18	0.20	0.19
SD	0.000	0.010	0.000	0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.000	0.005	0.000
% RSD	0.0	6.1	0.0	2.1	0.0	0.0	0.0	0.0	0.0	0.0	2.7	0.0	2.6	0.0
Overall % Mean	0.17		0.20		0.19		0.20		0.19		0.18		0.19	
Overall SD	0.007		0.005		0.000		0.005		0.000		0.005		0.005	
Overall % RSD	4.2		2.6		0.0		2.6		0.0		2.7		2.6	

MP: Method Precision *IP: Intermediate Precision*

CONCLUSION:

In this study, an HPLC technique for the simultaneous measurement of taurine, bilirubin, and major Bile acids in artificial Calculus Bovis was effectively established and developed. Regarding accuracy, precision, and linearity, the suggested approach was completely verified. The findings showed that the thorough methodology developed in this research was appropriate for the sensitive and exact determination of the contents of key components in artificial Calculus Bovis and may be utilised for the quality control of artificial Calculus Bovis and its manufactured products (20).

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