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Article

Validated UPLC Method for Determination of Unbound Bile Acids in Colesevelam HCl Tablets

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A simple, precise and accurate gradient reverse-phase ultra-performance liquid chromatographic method was developed for the quantitative determination of bile acids [glycocholic acid (GCA), glycochenodeoxycholic acid (GCDA) and taurodeoxycholic acid (TDCA)) in *in vitro* bile acid-binding study of Welchol tablets. The method was developed using Phenomenex Kinetex C18 (50 × 2.10 mm, 1.7 μm) column with mobile phase containing a gradient mixture of solvent A consisting of 0.02 M tetrabutylammonium phosphate (pH 7.5) and solvent B consists acetonitrile. The eluted compounds were monitored at 210 nm and the runtime was within 2 min. The binding parameter constants of Colesevelam HCl tablets 625 mg were determined using the Langmuir approximation at pH 6.8 by UPLC. The method is selective and capable of detecting bile acids in the presence of placebo matrix. The method has been validated with a lower limit of quantitation of 0.01 mM for bile acids. A linear response function was established for the range of concentrations 0.01–30.0 mM (*r* > 0.99) for GCA, GCDA and TDCA. The intra- and interday precision values for bile acids met the acceptance as per Food and Drug Administrations guidelines. The developed method was applied to *in vitro* bile acid-binding studies of Colesevelam HCl tablets.

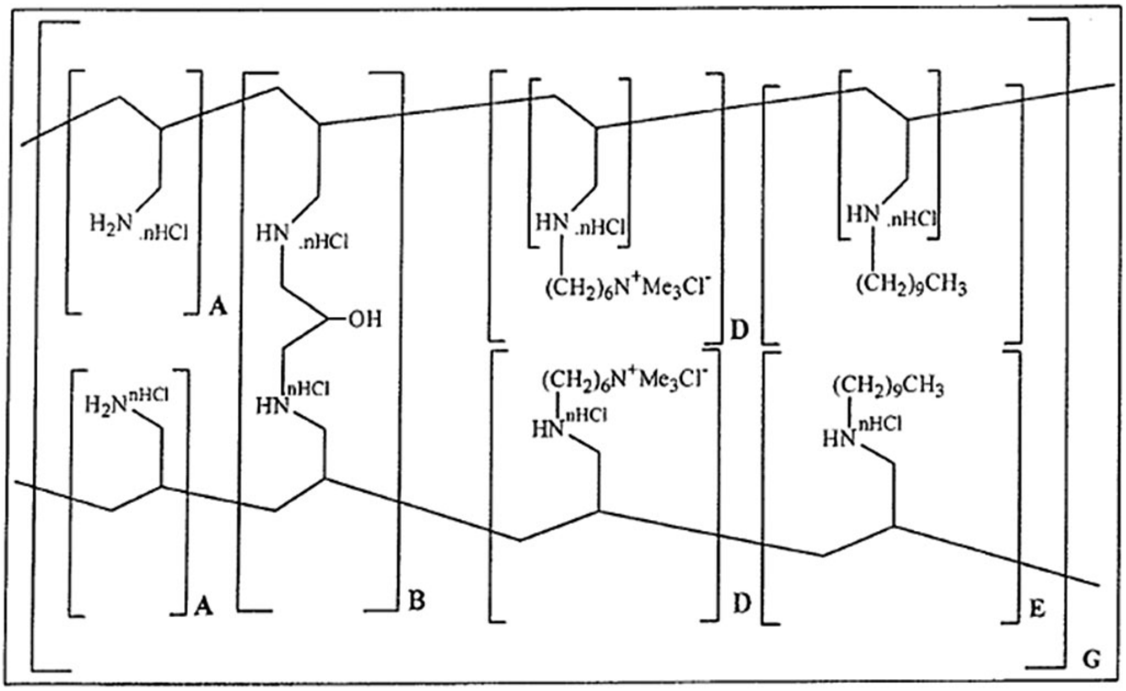
Introduction

Though high-performance liquid chromatography (HPLC) is a well-established reliable technique used in controlling the quality and consistency of active pharmaceutical ingredients and dosage forms; however, it is now described a slow technique in this modern technology. Ultra-performance liquid chromatography (UPLC) is a new category of separation technique based upon well-established principles of liquid chromatography, which utilizes sub-2 μm particles for stationary phase. These particles operate at elevated mobile-phase linear velocities to affect dramatic increase in resolution, sensitivity and speed of analysis. Owing to its speed and sensitivity, this technique is gaining considerable attention in recent years for pharmaceutical and biomedical analysis (1, 2). In this present work, this technology has been applied to the validation study of *in vitro* bile acid-binding method of Welchol tablets (Colesevelam HCl tablets).

Colesevelam Hydrochloride a non-absorbed, synthetic, lipid-lowering polymer, is a bile acid sequestrant. Colesevelam binds with high affinity to bile acids within the gastrointestinal (GI) tract, thereby inhibiting the re-absorption of bile acids, resulting in decreases in serum low-density lipoprotein cholesterol (LDL-C) levels. Colesevelam hydrochloride (Welchol, Daiichi Sankyo, Inc., Parsippany, NJ, USA) is a specifically engineered bile acid sequestrant polymer approved as adjunctive therapy to diet and exercise for the treatment of primary hypercholesterolemia (3–5). The structure of Colesevelam HCl is shown in Figure 1.

In clinical trials, Colesevelam HCl demonstrated an insignificant adverse event profile. The incidence of GI adverse events for patients treated with Colesevelam HCl was similar to that in patients treated with placebo. Colesevelam HCl is not absorbed from the intestine. In pharmacokinetic (PK) studies, Colesevelam HCl did not demonstrate clinically significant interactions with several drugs that are often prescribed concomitantly in patients who require cholesterol-lowering pharmacotherapy (e.g., lovastatin, fenofibrate, warfarin, digoxin and metoprolol). In contrast, conventional bile acid sequestrants have been shown to interfere with the PKs of several other drugs, including warfarin, thiazide diuretics, cardiac glycosides and propranolol (6–9). Because of the increased market potential of colesevelam for reduction of LDL-C in the setting of hyperlipidemia and for glycemic control in the setting of type 2 diabetes mellitus, patent expirations and proven concept of reduced costs with generic drug products, it is most likely that generic pharmaceutical manufacturers consider submission of abbreviated new drug applications for FDA approval. However, the generic pharmaceutical drug products have to conform to the same standards of quality, efficacy and safety required of innovator drug products. Testing procedures are to be established to prove that generic pharmaceutical drug products are therapeutically equivalent and interchangeable with their associated innovators product.

The bioequivalence of solid oral dosage forms intended for systemic delivery is established by *in vivo* PK endpoints such as C_{max} and AUC is neither appropriate nor feasible for testing the potency of locally acting tablets of colesevelam hydrochloride. For drugs whose site of action is the GI tract, determination of bioequivalence is more complicated as local drug concentrations cannot be measured directly. Therefore, it is a challenging issue for both the pharmaceutical industry and the regulatory agency to determine the BE of locally acting GI drugs. The guidance on bioavailability and bioequivalence drafted by Committee of Proprietary Medical Products of the European regulatory authorities stated 'for medicinal products not intended to be delivered into the general circulation, the common systemic bioavailability approach cannot be applied'. Draft guidance documents on locally acting GI drug products have been developed by FDA over the last several years to provide recommendations to sponsors to meet statutory and regulatory requirements (10–13). Generally, FDA addresses the issue on a case by case basis as outlined by the drug-specific guidance. Therefore, it is necessary to identify the key scientific principles for consistent and efficient identification of bioequivalence methods for locally acting



A= number of primary amine groups A= 0.14
B = number of cross-linked amine groups B= 0.12
D = monoquat alkylated amino groups D= 0.34
E = decylbromide alkylated amine groups E =0.40
G = Extended polymer chain.

Figure 1. Chemical structure of colesevelam HCl.

GI drugs. Generally, *in vitro*-binding studies are less variable, easier to control and more likely to detect differences between drug products if they exist. However, the clinical relevance of these *in vitro*-binding tests is vital for their validity.

The *in vitro* equilibrium-binding study procedures, described in the FDA guidance document, appear to be based on the pharmacological action of colesevelam hydrochloride. Colesevelam forms non-absorble complexes with bile acids in the GI tract and subsequently eliminated. Impairment of bile acid return leads to upregulation of hepatic bile acid synthesis through breakdown of cholesterol which results in an increased clearance of LDL-C. Thus, the clinical efficacy of colesevelam depends on its binding capacity to intestinal bile acids. The *in vitro*-binding capacity of colesevelam hydrochloride with sodium salts of glycocholic acid (GCA), glycochenodeoxycholic acid (GCDA) and taurodeoxycholic acid (TDCA) (Fig. 2) is an alternative approach to evaluate the efficacy of the locally acting colesevelam drug products. The *in vitro* equilibrium-binding study is considered as pivotal bioequivalence study for colesevelam hydrochloride tablets. The kinetic-binding study is used to support the pivotal equilibrium- and kinetic-binding capacities of Colesevelam hydrochloride with bile acids (GC, GCDA and TDCA) have been determined to compare the efficacy of two colesevelam tablet products according to the FDA guidance.

Determination of bile acids is a challenging job, since unbound bile acids found to be very low concentrations in *in vitro*-binding studies. Sensitive analytical technique needs to be developed and also method shall be capable of running bigger analytical batch runs without any frequent failures. We found an HPLC technique to estimate the unbound bile acids in the study of *in vitro* bile acid-binding study of Welchol tablets (14). However, the available literature method runtime is found to be 10 min, whereas we developed a sensitive UPLC method with a runtime of 2.0 min which is five times lesser than the literature method. The developed method can minimize expensive solvents being used for mobile-phase preparation; on the other hand, it limits the environmental pollution. The HPLC method runtime was $>$10 min (Fig. 3), which is significantly higher runtime when compared with UPLC method. This enabled us to develop a shorter runtime on UPLC. The developed method can quantitate the bile acids, i.e., GCA sodium, glycochenodeoxycholic acid sodium (GCDA) and taurodeoxycholic acid sodium (TDCA).

Experimental

Chemicals

Colesevelam HCl was obtained from Shasun Pharmaceuticals Ltd. (Chennai, India), potassium phosphate monobasic (KH_2PO_4), hydrochloric acid and 1 N aqueous sodium hydroxide were obtained from Spectrum Chemicals (New Brunswick, NJ, USA). Sodium hydroxide pellets were from Spectrum Chemicals

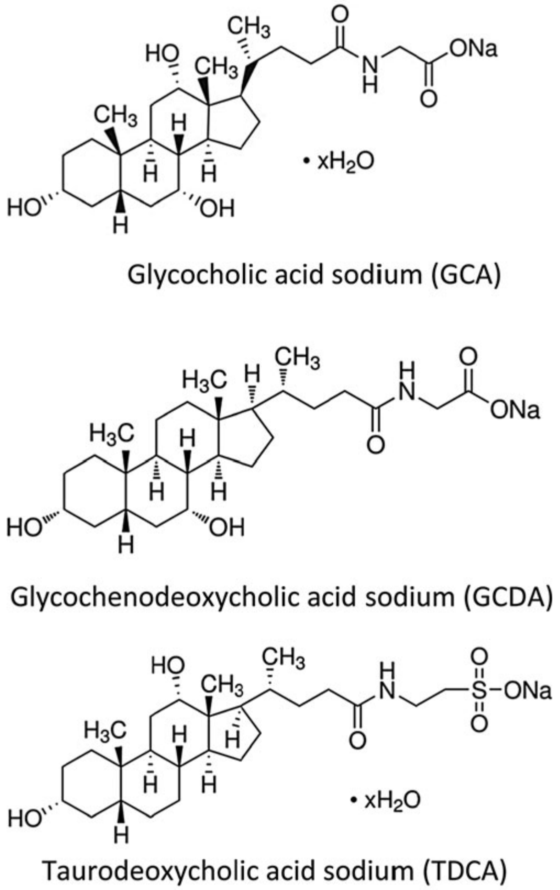


Figure 2. Chemical structures of bile acids.

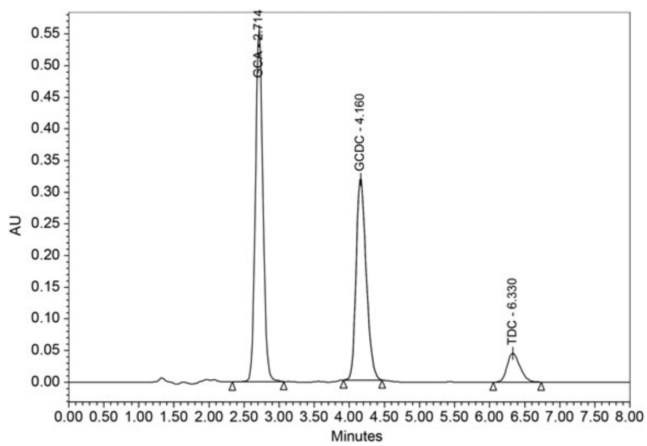


Figure 3. Chromatogram of bile acids on HPLC.

(New Brunswick, NJ, USA). GCA sodium, glycochenodeoxycholic acid sodium and taurodeoxycholic acid sodium were obtained from Sigma–Aldrich, USA. Deionized water was obtained from an in-house TOC water system (Sievers, USA). Welchol tablets were purchased in USA which was manufactured by Daiichi Sankyo (Parsippany, NJ, USA). Colesevelam HCl tablets 625 mg from InvaGen Pharmaceutical Inc. (Hauppauge, USA).

Apparatus

A Shimadzu Nexera UPLC system was used for the method validation and the study of *in vitro* bile acid binding. The system consists of LC-30AD pump, SIL-30AC auto sampler and CTO-30A column compartment and SPD-M30A PDA detector with lab solutions software control and data acquisition Version 5.3. Separations were performed using a Kinetex C18 analytical column (2.1 × 50 mm) 1.7 μm.

Chromatographic conditions

The method was optimized on gradient conditions. The Solvent A contains 0.02 M of tetrabutylammonium phosphate monobasic adjusted to pH 7.5 with 1 N sodium hydroxide and the Solvent B contains HPLC grade acetonitrile. The flow rate of mobile phase was 0.9 mL/min. The UPLC gradient program (time/B%) was set as 0.00/30, 0.80/60, 0.90/70, 0.95/80, 1.00/80, 1.20/80, 1.50/50 and 2.00/30. The column temperature was maintained ambient and the detection was monitored at a wavelength 210 nm. The injection volume was set to 2 μL.

Results

Specificity

Specificity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Placebo equivalent to 10 mg of colesevelam HCl was prepared in the simulated intestine fluid (SIF) and analyzed for possible interference of matrix with the analyte peaks, i.e., GCA, GCDA and TDCA. Refer the preparation of SIF in Discussion section. Standard solution was injected at 30 mM concentration, which is in the ratio of 3:3:1 of bile acids GCA, 17.14 mM, GCDA, 17.14 mM and TDCA, 5.72 mM. Standard solutions are also prepared in SIF solution. No interference (Fig. 4A and B) was observed from the PLACEBO matrix.

Calibration curve

The calibration curve for bile acid determination consists of a Calibration blank and six non-zero calibration standards covering concentrations at 0.01, 1.0, 7.0, 10.0, 20.0 and 30.0 mM of bile acids (GCA, GCDA and TDCA). These standards are incubated for 24 h at 37.0°C since colesevelam HCl tablets were incubated in the study of *in vitro* bile acid binding. Hence, the validation also conducted in similar fashion. Analyte peak areas were used to set up the calibration curve and to determine QC sample concentrations. Linear regression with linearity through zero was used to obtain the best fit of the data for the calibration curve. The acceptance criteria for each back-calculated standard concentration were ±15% deviation from the nominal value except an lower limit of quantification (LLOQ), which was set at ±20%. Calibration curve standards and quality control samples met the acceptance criteria for all experiments used in the final data, demonstrating satisfactory performance of the method during validation (Table I).

Precision and accuracy

The intra-assay precision and accuracy were estimated by analyzing replicates of bile acid solutions at four different QC levels, i.e., 0.01 (LLOQ), 0.03 low quality control (LQC), 4.0 middle quality control (MQC) and 10.0 high quality control (HQC) mM. The

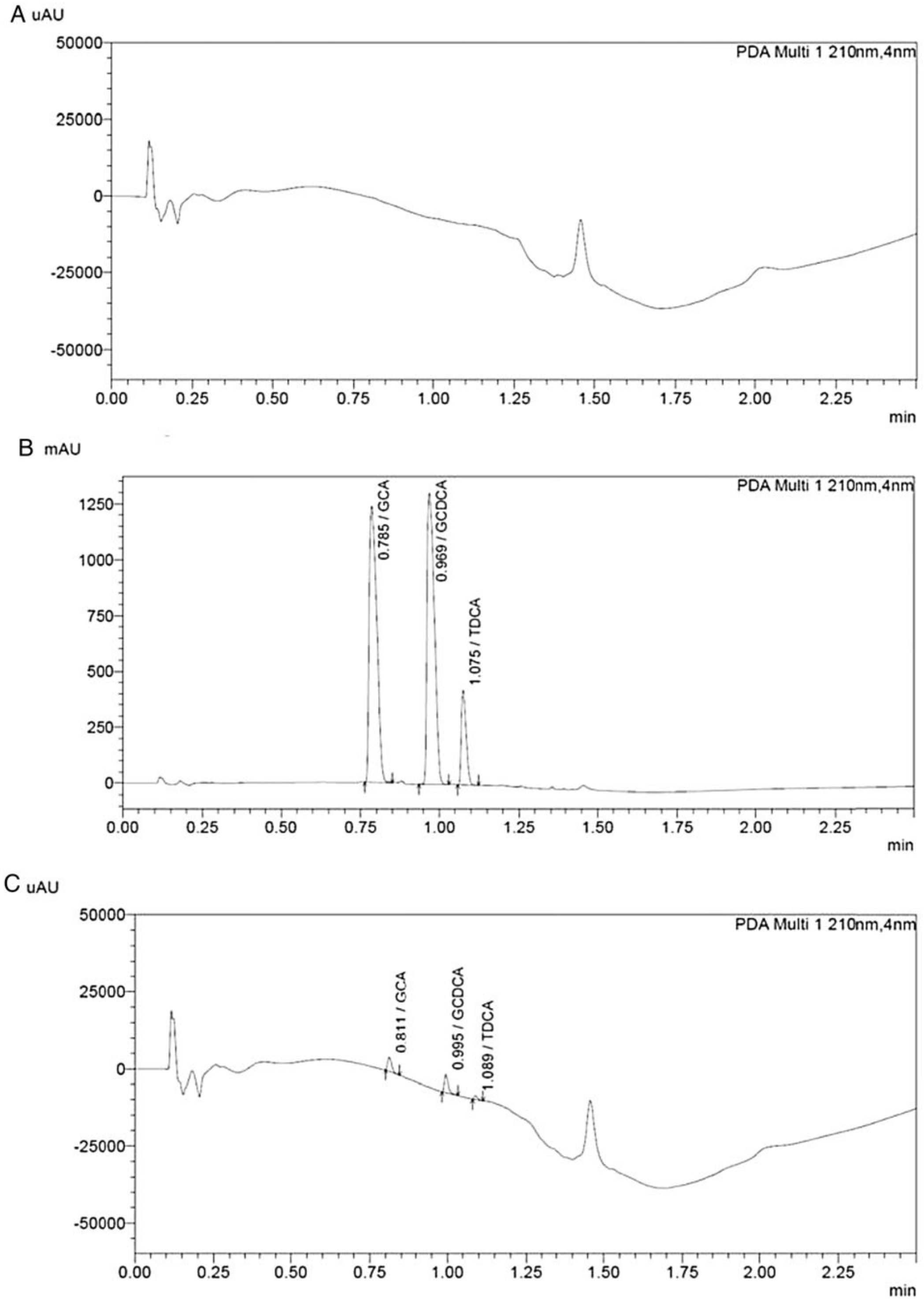


Figure 4. (A) Typical placebo chromatogram generated during the validation study. (B) Typical chromatogram of 30 mM bile acids standard solution. (C) LOQ chromatogram at 0.01 mM bile acids during the method validation.

interassay precision was determined by analyzing the four levels QC samples on three different runs. The criteria for acceptability of the data included accuracy between 85 and 115%, whereas LLOQ it should be between 70 and 130% and a precision of within $\pm$15% relative standard deviation (RSD) except for LLOQ, where it should not exceed 20% RSD (Table II).

Limit of quantitation

The lowest standard on the calibration curve is accepted as the limit of quantification. Prepared six independent LLOQ solutions of bile acid salts at 0.01 mM in SIF. Analyze the samples as per experimental conditions to calculate the concentration of LLOQ solutions (Fig. 4C).

Table I
Linearity Data for Bile Acids Generated During Method Validation

Day	Parameter	GCA	GCDA	TDC	Total bile acids (GCA + GCDA + TDC)
Day 1	Slope	291,229	281,829	193,874	273,292
	Correlation Coefficient	0.997857	0.998059	0.999941	0.998300
Day 2	Slope	287,196	278,645	189,501	269,575
	Correlation Coefficient	0.998040	0.997814	0.999855	0.998344
Day 3	Slope	296,924	286,475	193,874	277,724
	Correlation Coefficient	0.997639	0.996885	0.999941	0.997735
Day 4	Slope	316,428	287,530	203,116	287,856
	Correlation Coefficient	0.999689	0.995702	0.999631	0.998820
Day 5	Slope	314,684	332,396	198,317	305,651
	Correlation Coefficient	0.998876	0.999395	0.998973	0.999218
Day 6	Slope	304,818	316,645	201,806	295,171
	Correlation Coefficient	0.997071	0.997777	0.999490	0.998288
Average values of slope and correlation coefficient of 6 days of bile acids					
	Slope	301,880	297,253	196,748	284,878
	Correlation Coefficient	0.998195	0.997605	0.999639	0.998451

Table II
Intra- and Interday Precision Determination Quality Control Samples

Theoretical concentration (mM)	Run	Average measured concentration (mM)	RSD	Accuracy (%)
Intraday variation (six replicates at each concentration)				
0.01	1	0.00989	2.69	98.9
	2	0.01006	2.17	100.6
	3	0.00993	2.90	99.3
0.03	1	0.02985	2.41	101.6
	2	0.03047	2.80	101.6
	3	0.03049	2.13	101.6
4.0	1	4.05855	1.06	100.3
	2	4.03757	2.39	100.9
	3	4.09178	2.21	102.3
10.0	1	10.11195	1.94	101.1
	2	10.11755	2.60	101.2
	3	10.08285	3.22	100.8
Interday variation (18 replicates at each concentration)				
0.01		0.00996	2.6	99.6
0.03		0.03027	2.4	101.6
4.0		4.06263	1.9	101.2
10.0		10.1041	2.6	101.0

Stability

The benchtop stability was determined by analyzing six tablets of colesevelam HCl tablets were incubated in low (0.1 mM) and high (30.0 mM) concentrations of bile acids (GCA, GCDA and TDCA) which are prepared in SIF. As per the experimental conditions, samples were analyzed initially (0 h), after 24 and 48 h with freshly prepared calibration standards and two replicates of each low, medium and high QC's.

Robustness

Robustness is defined as degree of reproducibility of results obtained when various experimental conditions were altered such as rotation speed of incubator (RPM) and temperature. Robustness is established by analyzing three tablets of colesevelam HCl incubated in low (0.1 mM) and high (30 mM) concentrations of bile acids by altering the RPM (260, 280 and 300), temperature (34.0°C, 37.0°C and 40.0°C). The free bile acids are estimated with freshly prepared calibration standards and two sets of QC's.

Robustness study is also performed altering the pH (± 0.2) of the Solvent A and flow rate (± 0.1) of the mobile phase. Replicate injections of the bile acid standards are performed and system suitability parameters are evaluated to assess the robustness of the method. In all the cases, system suitability criteria have been satisfied.

Table III
Composition of Incubation Mixtures and Concentrations of Individual Bile Acid Salts at Various Target Concentrations in *In vitro* Bile Acid-Binding Study

Target (mM)	Stock (mL)	SIF (mL)	GCA (mM)	GCDA (mM)	TDCA (mM)
0.1	0.025	7.975	0.0428	0.0428	0.0143
0.3	0.075	7.925	0.1286	0.01286	0.0429
1.0	0.250	7.750	0.4285	0.4285	0.1428
3.0	0.750	7.250	1.2855	1.2855	0.4284
7.0	1.750	6.250	2.9995	2.9995	0.9996
10.0	2.500	5.500	4.2850	4.2850	1.4280
20.0	5.000	3.000	8.5700	8.5700	2.8560
30.0	7.500	0.500	12.855	12.855	4.2840

Discussion

Method development

The main objective of the chromatographic method was to separate the bile acids without compromising on its sensitivity and the runtime of the method. Initially, experiments were carried out using X Select CSH C18 (4.6 $\times$ 150 mm) 1.7 μm column using 0.08 M phosphate buffer (pH 3.0) as Solvent A and acetonitrile as Solvent B as a linear gradient program time/B%, 0.00/30, 0.80/60, 0.90/70, 0.95/80, 1.50/50 and 2.00/30. In these conditions, GCA and GCDA peaks were co-eluted. In next trial, column changed to X Bridge BEH Shield RP18 (2.1 $\times$ 50 mm) 1.7 μm by keeping the mobile-phase conditions unchanged. However, the separation was not seen between GCA and GCDA in these conditions. Better separation was observed when gradient conditions are optimized as time/B% 0.00/30, 0.80/40, 0.90/50, 0.95/60, 1.50/50 and 2.00/30. In these conditions, peak shape was not symmetric and even poor sensitivity was observed. It was found that use of 0.02 M tetra butyl ammonium phosphate buffer (pH 7.5) as Solvent A and acetonitrile as Solvent B with gradient elution was set as time/B%, 0.00/30, 0.80/60, 0.90/70, 0.95/80, 1.50/50 and 2.00/30 using different C18 UPLC columns (X Select CSH C18 4.6 $\times$ 100 mm, 1.7 μm, $\times$ Bridge BEH Shield RP 18 50 $\times$ 2.1 mm, 1.7 μm and Kinetex C18 50 $\times$ 2.10 mm, 1.7 μm) give increased responses of bile acids with better separation. Among all the columns, Kinetex column give better separation and increased sensitivity of bile acids. Finally, the mobile-phase conditions were optimized as mentioned in the Chromatographic conditions section.

In vitro bile acid-binding study

Equilibrium binding

Simulated intestine fluid preparation: 0.05 M phosphate buffer solution without enzyme which is adjusted to pH 6.8 with dil.NaOH.

Blank incubation mixture: Drug product equivalent to 10 mg was transferred into a flask and added 2 mL of SIF and soak at room temperature overnight. The next day added 8 mL of SIF to the blank.

Stock solution of bile acid salts in SIF: 40 mM of bile acids are prepared in SIF in the molar proportion 3:3:1. GCA sodium, 17.14 mM, GCDA, 17.14 mM and TDCA, 5.72 mM.

Preparation of samples without acid treatment: Individual tablets are crushed into powder and equivalent of 10 mg drug is transferred to eight incubation flasks of the Welchol tablets and colesevelam HCl tablets. Added 2 mL of SIF and soaked at room temperature overnight. The following day, added to each container the requisite volumes of SIF and 40 mM bile acids salts solution to make the final volume to 10 mL with SIF to achieve the target concentrations of bile acid salts covering the ranges of 0.1–30 mM. (Table III).

Preparation of samples with acid treatment: Individual tablets are crushed into powder and equivalent of 10 mg drug is transferred to eight incubation flasks of the Welchol tablets and colesevelam HCl tablets. Soaked the drug product in 0.1 N hydrochloric acid at 37°C for 1 h. Centrifuged and aspirated the supernate solution. Washed the drug product with SIF until (pH 6.8) is attained then follow the procedure under without acid treatment.

Standards solutions of bile acid salts: Diluted the requisite volumes of 40 mM stock solution of bile acid salts with SIF to yield 10 mL standard solutions of the following concentrations, i.e., 0.01, 1.0, 7.0, 10.0, 20.0 and 30.0 mM. The LQC (0.03 mM), MQC (4.0 mM) and HQC (10.0 mM) were prepared from separate stock solution of bile acid (40 mM).

All the standard and sample solutions were incubated at 37°C for 24 h. Filtered and collected filtrate used for the analysis to determine the unbound bile acid content.

Data treatment and analysis: The amount of bile acid salt bound to colesevelam HCl is obtained from the difference between the initial concentration of bile acid salt introduced into the system and the concentration present in the filtrate at the end of the study. The monomolecular adsorption of adsorbate (bile acid salt) molecules from solution, at constant temperature, on to an adsorbent (colesevelam HCl) is described by the following Langmuir-type equation:

$$\frac{C_{eq}}{(x/m)} = \frac{1}{k_1 k_2} + \frac{C_{eq}}{k_2}.$$

where

C_{eq} concentration of the adsorbate (bile acid salt) remaining in the solution at equilibrium;
X the amount of adsorbate bound to the adsorbent (colesevelam HCl);
M the amount of adsorbent used;

The constant k_1 is defined as the adsorption coefficient or affinity constant and is related to the magnitude of the forces involved in the binding process.

The Langmuir capacity constant k_2 indicates the apparent maximum amount of adsorbate that can be adsorbed per unit weight of adsorbent.

From the concentration of bile acid salt in the solution at equilibrium, the amount of bile acid salt, expressed in micromoles and as a percentage, bound to 10 mg of colesevelam HCl was calculated. Form this amount of bile acid salt bound per mg of drug, the relationship x/m is calculated. A plot of $C_{eq}/(x/m)$ versus C_{eq} on rectilinear coordinates should yield a straight line. Application of regression analysis will yield a slope (a) and intercept (b) of the line. The affinity constant k_1 and capacity constant k_2 are calculated from the slope and intercept. The k_1 value

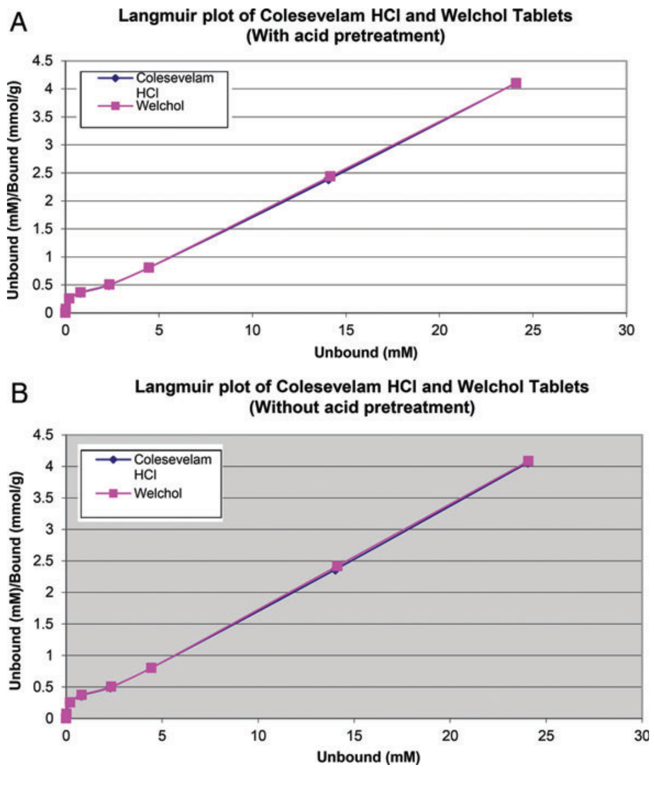


Figure 5. (A) Langmuir plot of colesevelam HCl tablets 625 mg and Welchol tablets 625 mg with acid treatment in *in vitro* bile acid-binding study. (B) Langmuir plot of colesevelam HCl tablets 625 mg and Welchol tablets 625 mg without acid treatment in *in vitro* bile acid-binding study.

Table IV
The Affinity and Langmuir Capacity Constants between Colesevelam HCl Tablets 625 mg (InvaGen) and Welchol Tablets (Brand) 625 mg

Buffer solutions at pH 6.8	Colesevelam HCl tablets 625 mg		Welchol tablets 625 mg		k_1 (slope/intercept)			k_2 (1/slope)	
	Slope	Intercept	Slope	Intercept	InvaGen (*T*)	Brand (*R*)	*T*/*R* ratio	InvaGen	Brand
With acid	0.164	0.120	0.165	0.123	1.367	1.333	102.55	6.098	6.098
Without acid	0.163	0.116	0.164	0.119	1.405	1.378	101.96	6.135	6.098

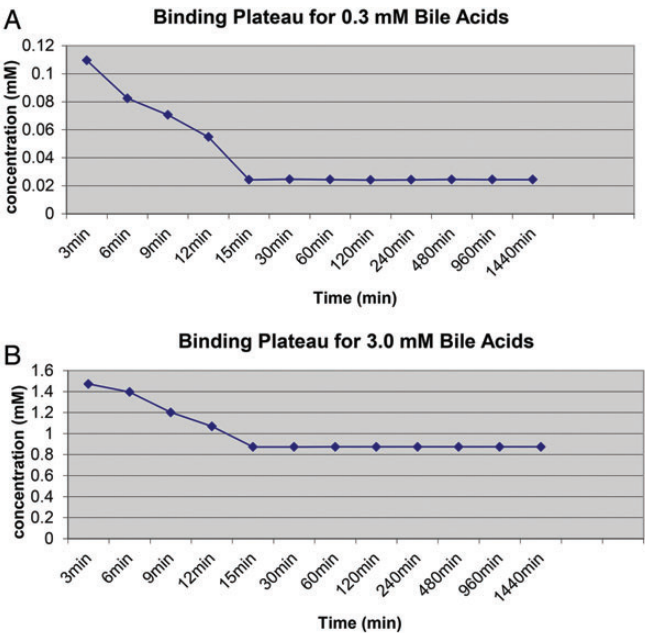


Figure 6. (A) Graphical representation of concentration of bile acids versus time at 0.3 mM bile acid-binding plateau. (B) Graphical representation of concentration of bile acids versus time at 3.0 mM bile acid-binding plateau.

is calculated by dividing the slope of the regression line by the intercept, the k_2 value is equal to the inverse of the slope. The affinity and binding constants were found to be similar in colesevelam HCl tablets and Welchol tablets (Fig. 5A and B and Table IV).

Binding plateau

The study was performed by incubating 10 mg equivalent of colesevelam HCl tablets in 0.3 and 3.0 mM bile acid salts (GCA, GCDA and TDCA) as per the experimental conditions. Each 10 mL samples of bile acid salts (GCA, GCDA and TDCA) were withdrawn from incubator vessel at time intervals of 15, 30, 60, 120, 240, 480, 960 and 1440 min's. The samples were filtered through 0.45 μm filters and analyzed using UPLC.

The data obtained were graphically represented for free/bound bile acid salts (Fig. 6A and B). Based on the graph, it is concluded that maximum binding was achieved at 15 min.

Conclusions

A validated UPLC method has been developed to estimate unbound bile acids in colesevelam HCl tablets. The method is selective and is capable to detect unbound bile acids quantitatively in the binding study. The method has been validated in terms of specificity, precision, linearity, accuracy, limit of quantification and robustness. The validated method can be used to estimate unbound bile acids in *in vitro* studies of colesevelam HCl tablets. The determinations of binding parameter constants were compared between Welchol tablets and colesevelam HCl tablets and results are in good agreement.

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