International Journal of Advance and Applied Research
www.ijaar.co.in
ISSN – 2347-7075 Impact Factor – 7.328
Peer Reviewed Bi-Monthly
Vol.10 No.6 July – August 2023

HPLC-Based Method Development Of Bile Acid With Precolumnderivatization

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DOI - 10.5281/zenodo.8353952

Abstract:

Hepatic enzymes synthesize bile acids from cholesterol and excrete them into the small intestine through the bile duct. They regulate cholesterol homeostasis, lipid absorption, medication and vitamin excretion, and toxin recirculation. For protein biopharmaceutical analysis, Biles and amino acid precolumn and Postcolumn derivatization methods have been developed and confirmed. In a general-purpose UV-visible HPLC system, protein hydrolysis, derivatization, and reversed-phase HPLC analysis are performed. Biles and amino acids (aspartic acid, glutamic acid, serine, glycine, glutamine, threonine, alanine, histidine, tyrosine, valine, methionine, leucine, isoleucine, phenylalanine, tryptophan, lysine, proline, Selenocysteine, Arginine, Asparagine, the validation parameters specificity, linearity, accuracy, precision, limit of detection, and limit of quantification were examined. The approach can determine recombinant protein extinction coefficients with absolute measurement.

Keywords: Bile acids, Amino acids, HPLC, Development, Validation.

Introduction:

Perhaps the most common application of precolumn derivatization methods is the transformation of pharmaceuticals into compounds that absorb ultraviolet light. During the early phases of the development of HPLC, when single-wavelength detectors were utilised, it became immediately evident that pharmacological compounds needed to be converted into species capable of absorption at 254 nm. However, the introduction of multi-wavelength detectors and photo-diode array detectors (PDA) has reduced the conversion of pharmaceuticals to UV-active components. Artemisinins, which lack the appropriate chromophore, are obviously the sole exception to this norm (1-3).

Previously utilised UV-derivatization techniques prior to LC separation are described below. Petsch et al. (2004) outlined a method for the accurate measurement of dithiocarbamates in plasma samples by employing a fully automated precolumn derivatization procedure. To complete the complexation procedure, a portion of the sample was mixed with a portion of a 5 mM Co (II) solution that was placed on the auto

sampler tray. To attain optimal conditions, the reaction was carried out in the receptacle for 11 minutes at 20 degrees Celsius. Using a C18 ODS column, a binary gradient of phosphate buffer and methanol was utilised to complete the LC separation procedure. The vibrant mixture of dithiocarbamate and Co (III) was identified using UV detection at a wavelength of 330 nm. Using this assay, pyrrolidine dithiocarbamate, a potent antiviral medication for the suppression of human Adegoke 130 rhinoviruses and influenza viruses, was discovered in plasma samples. In 1986, Shishan and Mei-Yi were the first to describe the Quinhaosu pre-column reaction and a method for identifying it in animal plasma (4-6).

Technology was utilised to accomplish both of these objectives. In order to convert quinhaosu into the UV-absorbing substance Q260, it was initially treated with 0.16% NaOH at 45°C for 30 minutes. Then, acetic acid was utilised to acidify the final product. Conditions considered most advantageous for the pre-column reaction were investigated. The recommended solvent for isolating plasma samples was ethyl acetate. After evaporating the quinhaosu from the residue, it was converted to 0260 using a precolumn reaction, and its concentration was determined using HPLC. Since all artemisinins require UV activation in some fashion, pre-column derivatization of artemisinin derivatives is especially advantageous. Adegoke et al. (2012) used pre-column derivative synthesis and the 4-carboxyl-2, 6-dinitrobenzene diazonium ion to document the reaction of artemisinin and its three commonly used derivatives (articulate, dehydroartemisinin, and Artemether). This reaction exhibited a high degree of sensitivity and was straightforward to reproduce. Using a chemical technique that converts amino acids into UV-absorbing species, the order of amino acids in proteins has been determined successfully for some time. Literature acknowledges the use of compounds in the pre-column derivatization of amino acids (7, 8).

Material And Method:

Trial 1:

According to the study, "Kakiyama G, Muto A, Takei H, Nittono H, Murai T, Kurosawa T, Hofmann AF, Pandak WM, and Bajaj JS. A straightforward and precise HPLC method for faecal bile acid profile in healthy and cirrhotic individuals: validation by GC-MS and LC-MS [S]. "Journal of Lipid Research, 1 May 2014, Volume 55, Issue 5:978-90." Cholic acid (CA), Taurocholic acid (TA), Taurochenodeoxycholic acid (TCDA), Glycocholic acid (GA), Glycochenodeoxycholic acid (GDA), Deoxycholic acid (DA), and chenodeoxycholic acids (CDA) were analyzed and compared to other Bile acids.

Consequently, new method development for Related Substances testing was initiated by citing the above research paper (9-11).

Chromatographic Conditions:

Instrument	:	HPLC
Column	:	YMC Pack ODS-AM, 150 x 4.6 mm, 3µm
Injection	:	20 µL
Flow rate	:	1.0 mL/min
Wavelength	:	UV 205 nm
Column Temperature	:	40°C
Sample Temperature	:	25°C
Run Time	:	37 minutes
Needle wash	:	Mixture of Acetonitrile and Water in the ratio of 50:50.
Seal wash	:	Mixture of Acetonitrile and Water in the ratio of 10:90.

Gradient Program:

Table (a) showing the gradient Gradient Program for Trial 1 in bile acids (Precolumn Derivatization)

Preparation of Solutions:

Method A: Without alkaline hydrolysis step:

Before being utilised, the lyophilized faeces were finely ground into a powder. In a screw-top glass tube, 10–20 mg of pulverized defecation were suspended in 250 ml of cool water and heated for 10 minutes at 90 °C. Using an ultrasonic bath, any remaining large particulates were dispersed following heating. The mixture was then incubated for 16 hours at 37 degrees Celsius with a sodium acetate buffer (100 mM, pH 5.6; 250 µl) containing 15 units of cholylglycine hydrolase and 150 units of sulfatase. The reaction was terminated after adding 250 µl of isopropanol and heating the mixture for 10 minutes at 90 °C. Included were 50 nmol of norDCA, 3 ml of 0.1 N NaOH, and an internal standard (IS). The bile acids were extracted by ultrasonically disrupting the faecal matrix for one hour at room temperature using a Branson type B-220 ultrasonic chamber (Danbury, CT). After centrifugation, the particle was washed with 0.1 N NaOH (2 ml), and the supernatant was transferred to a glass test tube. The combined extract was introduced into a Waters Sep Pak tC18 cartridge (500 mg sorbent) primed with methanol (10 ml) and water (10 ml). Water (5 ml), 15% acetone (4 ml), and water (5 ml) were used in succession to clean the cartridge. After being eluted with 6 ml of methanol, retained bile acids were evaporated to dryness under a N2 stream at a temperature below 40°C (12, 13).

Method B: with alkaline hydrolysis step.

After the cholylglycine hydrolase/sulfatase treatment (method A), 500 µl of isopropanol and 100 µl of 1 N NaOH were added to the solution, which was then incubated at 60°C for 2.5 hours. Adding an IS, 50 nmol of norDCA, and 3 ml of 0.1 N NaOH, the bile acids were extracted in the same manner as described previously (14).

Extracted unconjugated bile acids (either by method A or B) wererederivatized with 24 –phenacyl ester as [derivatizing agent]

Trial 2:

To Glycochenodeoxycholic acid (GDA), Deoxycholic acid (DA) and chenodeoxycholic acids (CDA)peak by gradient Study. For other details refer above trial 1

Gradient Program:

Table (b) showing the gradient Gradient Program for Trial 2 in bile acids (Precolumn Derivatization).

Trial 3:

Gradient study to improve baseline pattern. For other details refer above trial 1 Flow rate: 1.0mL/min. Column oven temperature: 40°c

Gradient Program:

Table (c) showing the gradient Gradient Program for Trial 3 in bile acids (Precolumn Derivatization).

Trial 4:

Gradient study to separate Glycochenodeoxycholic acid (GDA), and chenodeoxycholic acids (CDA)peaks. For other details refer above trial 1

Flow Rate: 1.0mL/min. Column oven temperature:40°c

Gradient Program:

Table (d) showing the gradient Gradient Program for Trial 4 in bile acids (Precolumn Derivatization).

Trial 5:

Gradient study, mobile phase to separate Glycochenodeoxycholic acid (GDA), and chenodeoxycholic acids (CDA)peak (15).

Chromatographic Conditions:

Instrument	:	HPLC
Column	:	YMC Pack ODS-AM, 150 x 4.6 mm, 3µm
Injection	:	20 µL
Flow rate	:	1.0 mL/min
Wavelength	:	UV 205 nm
Column	:	45°C
Sample	:	25°C

Gradient Program:

Table (e) showing the gradient Gradient Program for Trial 5 in bile acids (Precolumn Derivatization).

Preparation of Solutions:

Dilute Orthophosphoric acid for buffer pH adjustment preparation:

Transfer and thoroughly combine 10.0 mL of Orthophosphoric acid into a 100 mL volumetric vial containing approximately 200 mL of water. Mix and dilute with water to the desired volume (16).

Buffer solution pH 2.5:

Dissolve approximately 3.45 grammes of sodium dihydrogen phosphate monohydrate in 1000 milliliters of milli Q water. Adjust the pH to 2.5 ± 0.05 with phosphoric acid diluted in water while agitating. Stop agitating and observe the pH for 5 to 10 minutes; if necessary,

regulate the pH. Utilise a 0.22 µm PVDF membrane filter for filtration.

Mobile phase A:

Use 100% Buffer solution pH 2.5 as Mobile Phase A

Mobile phase B:

Use 100% Acetonitrile as Mobile Phase B

Trial 6:

Gradient study and column oven temperature study to enhance baseline pattern at the elution of Glycochenodeoxycholic acid (GDA) and chenodeoxycholic acid (CDA)peak (17).

Chromatographic Conditions:

Instrument	:	HPLC
Column	:	YMC Pack ODS-AM, 150 x 4.6 mm, 3µm
Injection	:	20 µL
Flow rate	:	1.00 mL/min
Wavelength	:	UV 205 nm
Column	:	50°C
Sample	:	25°C

Gradient Program:

Table (f) showing the gradient Gradient Program for Trial 6in bile acids (Precolumn Derivatization).

Preparation of Solutions:

Dilute Orthophosphoric acid for buffer pH adjustment preparation:

Transfer and thoroughly combine 10.0 mL of Orthophosphoric acid into a 100 mL volumetric vial containing approximately 200 mL of water. Mix and dilute with water to the desired volume (18, 19).

Buffer solution pH 2.5:

Dissolve approximately 3.45 grammes of sodium dihydrogen phosphate monohydrate in 1000 milliliters of milli Q water. Adjust the pH to 2.5 ± 0.05 with phosphoric acid diluted in water while agitating. Stop agitating and observe the pH for 5 to 10 minutes; if necessary, regulate the pH. Utilise a 0.22 µm PVDF membrane filter for filtration.

Mobile phase A:

Use 100% Buffer solution pH 2.5 as Mobile Phase A

Mobile phase B:

Use 100% Acetonitrile as Mobile Phase B

Result And Discussion:

Trial 1:

Observations:

- Cholic acid (CA), Taurocholic acid (TA), Taurochenodeoxycholic acid (TCDA), Glycocholic acid (GA) peak eluted
- Glycochenodeoxycholic acid (GDA), Deoxycholic acid (DA) and chenodeoxycholic acids (CDA) peak not eluted (figure a).

Conclusion:

To separate Glycochenodeoxycholic acid (GDA), Deoxycholic acid (DA) and chenodeoxycholic acids (CDA) peak gradient study needs to be performed

Trail 2:

Observations:

- Peak of Cholic acid (CA), Taurocholic acid (TA), Taurochenodeoxycholic acid (TCDA), Glycocholic acid (GA) along with Glycochenodeoxycholic acid (GDA) was eluted.
- Deoxycholic acid (DA) and chenodeoxycholic acids (CDA) peak not eluted (figure b).

Conclusion:

To elute remaining peaks of deoxycholic acid (DA) and chenodeoxycholic acid (CDA), gradient programme analysis and mobile phase analysis are required.

Trial 3:

Observations:

- Deoxycholic acid (DA) peak was not eluted.
- The peak of Glycochenodeoxycholic acid (GDA), and chenodeoxycholic acids (CDA) eluted but merged in each other (figure c).

Conclusion:

Gradient program study, mobile phase study needs to be performed to elute Deoxycholic acid (DA) peak.

Trial 4

Observations:

- Deoxycholic acid (DA) peak was not eluted.
- The peak of Glycochenodeoxycholic acid (GDA), and chenodeoxycholic acids (CDA) eluted and separated well.
- Need to check baseline pattern (Figure d).

Conclusion:

Gradient program study, mobile phase study needs to be performed to eluate Deoxycholic acid.

Trail 5:

Observations:

- Deoxycholic acid (DA) peak was not eluted.
- Glycochenodeoxycholic acid (GDA), and chenodeoxycholic acids (CDA) peak eluted on inclined baseline pattern
- Need to correct baseline pattern (Figure e).

Conclusion:

Gradient program study, mobile phase study needs to be performed to correct baseline pattern and to elute Deoxycholic acid (DA) peak.

Final Method for low UV sensitive drugs like bile acids (Precolumn Derivatization)

Trail 6 (Final Method):

Observations:

• All summits It was possible to distinguish Cholic acid (CA), Taurocholic acid (TA), Taurochenodeoxycholic acid (TCDA), Glycocholic acid (GA), Glycochenodeoxycholic acid (GDA), and chenodeoxycholic acids (CDA).

• The resolution between the peaks of Glycochenodeoxycholic acid (GDA) and chenodeoxycholic acid (CDA) is adequate (Table g).

• The peak of deoxycholic acid (DA) was not eluted. This peak can be eluted during Postcolumn derivatization (Figures f and g).

Conclusion:

Hence this method is finalized for the validation of Cholic acid (CA), Taurocholic acid (TA), Taurochenodeoxycholic acid (TCDA), Glycocholic acid (GA), Glycochenodeoxycholic acid (GDA) and chenodeoxycholic acids (CDA).

Figures and Tables:

Figures:

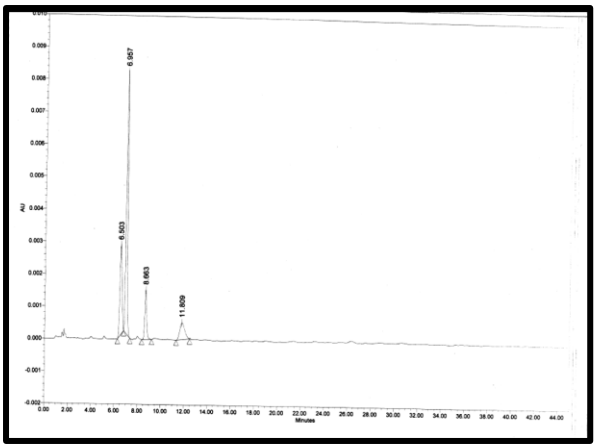

Figure (a) Chromatogram for trial 1 in Bile Acids (Precolumn Derivatization)

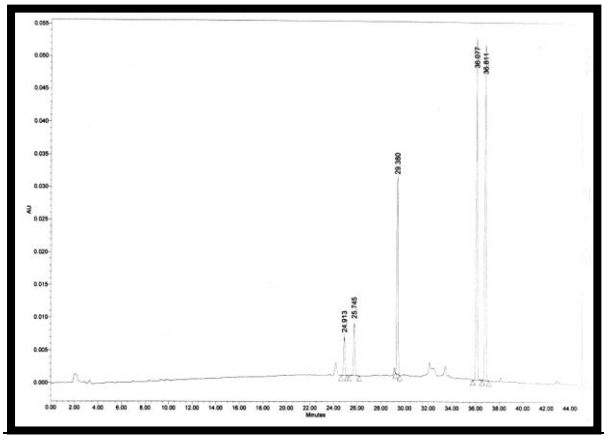

Figure (b) Chromatogram for trial 2 in Bile Acids (Precolumn Derivatization)

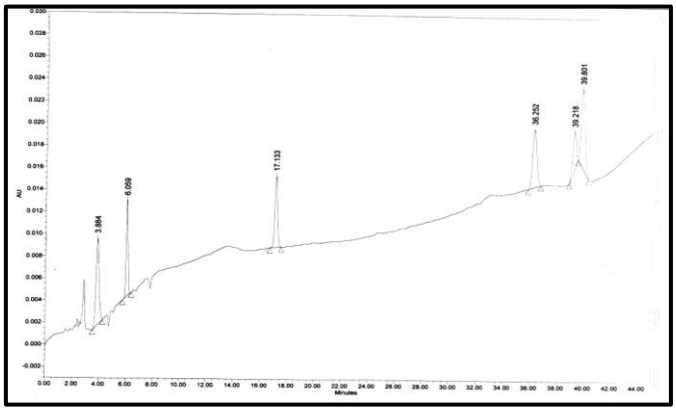

Figure (c) Chromatogram for trial 3 in Bile Acids (Precolumn Derivatization)

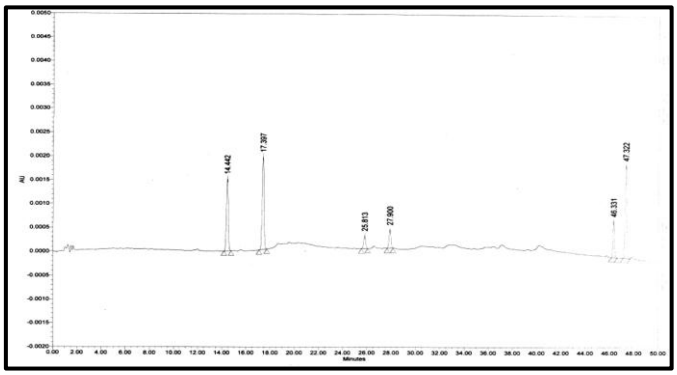

Figure (d) Chromatogram for trial 4 in Bile Acids (Precolumn Derivatization)

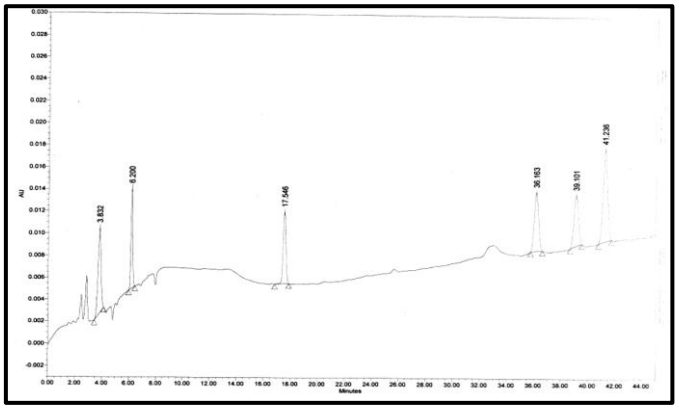

Figure (e) Chromatogram for trial 5 in Bile Acids (Precolumn Derivatization)

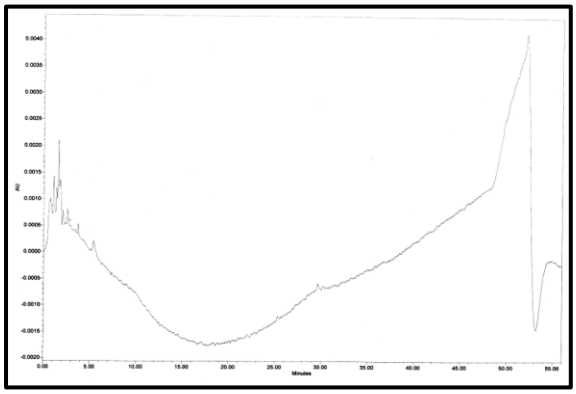

Figure (f) Blank Chromatogram for trial 6 in Bile Acids (Precolumn Derivatization)

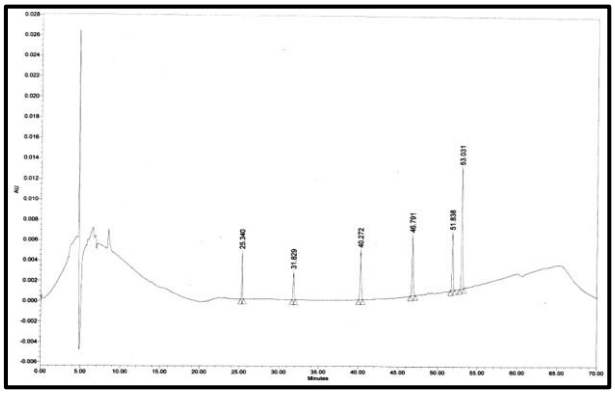

Figure (g) Sample Chromatogram for trial 6 in Bile Acids (Precolumn Derivatization)

Tables:

Table (a) Gradient Program for Trial 1 in bile acids (Precolumn Derivatization)

Time (min.)	Mobile Phase A (%)	Mobile Phase B (%)
0	87	13
2	87	13
25	46	54
25.1	25	75
29	25	75
29.1	87	13
45.0	87	13

Table (b) Gradient Program for Trial 2 in bile acids (Precolumn Derivatization)

Time (min.)	Mobile Phase A (%)	Mobile Phase B (%)
0	87	13
5	87	13
28	63	17
40	40	60
41	87	13
45	87	13

Table (c) Gradient Program for Trial 3bile acids (Precolumn Derivatization)

Time (min.)	Mobile Phase A (%)	Mobile Phase B (%)
0	87	13
5	87	13
15	65	35
35	25	75
37	87	13
45	87	13

Table (d) Gradient Program for Trial 4 in bile acids (Precolumn Derivatization)

Time (min.)	Mobile Phase A (%)	Mobile Phase B (%)
0	87	13
5	89	10
27	68	32
40	25	75
41	87	13
47	87	13

Table (e) Gradient Program for Trial 5 in bile acids (Precolumn Derivatization)

Time (min.)	Mobile Phase A (%)	Mobile Phase B (%)
0	90	10
24	68	32
40	38	62
42.1	90	10
48	90	10

Table (f) Gradient Program for Trial 6 in bile acids (Precolumn Derivatization)

Time (min.)	Mobile Phase A (%)	Mobile Phase B (%)
0	90	10
7	87	13
26	66	34
46	58	42
50	45	55
50.1	90	10
56	90	10

Table (g) Chromatographic reading for trial 6 in Bile Acids (Precolumn Derivatization)

Sr. no	Peak Name of bile acid	Retention time(min)	Area (Uvsec)	% Area	Height (Uv)	USP Resolution	UsSP tailing	USP Plate count
1	Taurocholic acid (TA)	25.340	32355	9.36	4472	17.20	1.0	278738
2	Cholic acid (CA)	31.829	24526	7.10	2587	29.10	1.0	258534
3	Taurochenodeoxycholic acid (TCDA)	40.272	51798	14.99	4698	30.70	1.0	258534
4	Glycocholic acid (GA)	46.791	60980	17.62	5840	22,70	1.1	483697
5	Glycochenodeoxycholic acid (GDA)	51.838	55766	16.14	5565	18.50	1.0	607415
6	Chenodeoxycholic acids (CDA)	53.031	120198	34.79	11835	4.4	1.0	611116

Conclusion:

The breakdown of cholesterol and lipids, as well as the participation of bile salts (BS) in the process, are two of the many important metabolic pathways. HPLC, or high performance liquid chromatography, is the method that is used the vast majority of the time for analyzing BS in a variety of samples. However, the existing HPLC analytical techniques utilised to analyse and quantify single BS in in vitro digested samples demonstrated inadequate separation of a complex combination of BS. These approaches were applied. A standard approach that was initially utilised for measuring individual BS in food samples that were exposed to in vitro digestion was enhanced by the authors of this research. In addition, we modified a technique that had been established in the past for the evaluation of BS in human blood samples so that it could be used to the study of these molecules in chyme samples that were acquired via a simulation of gastrointestinal digestion. Our approach was simple, and it was able to separate and quantify the four major BS (sodium salts of Glycocholic, taurocholic, Glycochenodeoxycholic, and Taurochenodeoxycholic acids) in a short amount of time (20).

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