ISSN 2063-5346

SIMULTANEOUS ESTIMATION OF BILE ACID WITH POST- COLUMN DERIVATIZATION BY USING HPLC METHOD

Pravin Dinkar Telgote

Research Scholar, Shri Jagdishprasad Jhabarmal Tibrewala University [JJTU], Vidyanagari, Jhunjhunu, Rajasthan- 333001.

Sanjay Kisanrao Bais

Shri Jagdishprasad Jhabarmal Tibrewala University, Vidyanagari, Jhunjhunu [JJTU], Rajasthan- 333001.

Article History: Received: 12.06.2023 **Revised:** 14.07.2023 **Accepted:** 31.07.2023

Abstract

Using UPLC coupled with triple quadrupole mass spectrometry (QqQ-MS) in the multiple reaction monitoring (MRM) mode and HPLC coupled with quadrupole time-of-flight tandem mass spectrometry (Q/TOF-MS) in the MS/MS mode, twelve bile acids in Bile Arisaema were analyzed qualitatively and quantitatively. The fragmentation route of seven bile acids has been postulated. Over a wide concentration range, the quantification technique showed outstanding linearity, repeatability, stability, accuracy, and recovery. Seven chemicals in Bile Arisaema have compositions that varied greatly by area. The fermentation period of bile acid was effectively adjusted using chemometric methods, hierarchical clustering analysis (HCA), and principal components analysis (PCA).

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

Introduction

Fluorescence emission and UV-visible absorbance should both be used as analytical methods for high or low pressure liquid chromatography (HPLC) examinations. High-performance liquid chromatography, more often referred to as HPLC, is a method that can be depended upon to determine the concentration of the analyte of interest. The high-performance liquid chromatography (HPLC) technique was selected as the method of detection because of its adaptability in terms of signal intensity, its user-friendliness, and its comparatively cheap cost. In order to get over roadblocks and cut down on interferences, chromatographers often resort to chromophores with strong absorption or fluorescence peaks as a means of converting chromatographically partitioned analytes into derivatives (1-3). This is done in order to improve the efficiency of the analysis. As a consequence of this, the analytes go through a process of conversion. In order to achieve chromatographic separation, post-

column derivatization (PCD) of analytical chemicals that are present in the column is performed (see Figure a). Post-column derivatization, often known as PCD, is a process that typically makes use of the chemicals Ninhydrin, fluorescamine, and o-phthalaldehyde, which are referred to together as OPA (4, 5). Fluorescein's effect on secondary amino acids is either insignificant or nonexistent, depending on the context. As a consequence of this, the calculations that include amines need just 10% more fluoresceine. The outcomes of the reaction that takes place between fluoresceine and the principal amino acids in a pH 9 buffer are analyzed using the assistance of an RP-HPLC fluorescence detector. Following the derivatization of the column, the amount of time required for the reaction between fluoresceine and the main amino acids was reduced by a few seconds, which led to a shorter overall length. According to the findings of research carried out by Moldoveanu and his colleagues, the PCD technique makes use of the separation reaction coil. The PCD makes use of Active Flow Technology (AFT) columns, which are among the most cutting-edge technology currently available in their sector (6-8).

Literature Review

By forming micelles that solubilize the cholesterol in the bile, bile acids, which are essential cholesterol constituents, make it simpler for the body to eliminate the substance via defecation (9). Bile acids are conjugated with the amino acids glycine and taurine in the liver before being deposited in the gallbladder and then discharged into the duodenum through the bile duct. Bile acids promote lipolysis and aid in the absorption of lipids, including fat-soluble vitamins, by interacting with lipases and producing mixed micelles in the intestinal lumen (10-12). During enterohepatic circulation, intestinal bacteria may modify the primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA), which are produced in the liver, into secondary bile acids, primarily deoxycholic acid (DCA), lithotomic acid (LCA), and ursodeoxycholic acid (UDCA). In healthy peripheral circulation and urine, bile acids are only present in trace quantities. In hepatobiliary and intestinal disease, however, disruptions in the liver's production and clearance of bile acids, as well as the intestine's absorption, will influence the concentration and distribution of bile acids in the blood (13). Thus, bile acid analysis may be useful for assessing abnormal liver or intestinal function and identifying associated diseases. Some of the conclusions that can presently be drawn from the research conducted on these individuals are ambiguous (14).

Research Methodology

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 (15).

Trial 2

Reagents, chemicals, filters and column:

Reagents, chemicals, filters, and column for HPLC-based simultaneous estimation of

bile acid with post-column derivatization are shown in Table (a).

Chromatographic conditions:

Instrument	:	HPLC
Column	:	YMC Pack ODS-AM, 150 x 4.6 mm, 3µm
Injection Volume	:	50 µL
Flow rate	:	1.20 mL/min
Wavelength	:	UV 205 nm
Column Temperature	:	50°C
Sample Temperature	:	25°C
Run Time	:	75 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.

Preparation of solutions

Method A: without alkaline hydrolysis step.

Before usage, the lyophilized faeces was thoroughly ground into a powder. Pounds of powdered faeces (10–20 mg, precisely weighed) were suspended in 250 ml of cold water and boiled at 90 °C for 10 minutes in a screw-capped glass tube. After heating, any remaining big particles were broken up using an ultrasonic bath. The mixture was then incubated at 37°C for 16 hours with a sodium acetate buffer (100 mM, pH 5.6; 250 µl) containing 15 units of cholylglycine hydrolase and 150 units of sulfatase. After adding 250 l of isopropanol and heating the mixture for 10 minutes at 90 °C, the reaction was stopped (16). 50 nmol of norDCA, 3 ml of 0.1 N NaOH, and an internal standard (IS) were included. By ultrasonically disrupting the faecal matrix for one hour at room temperature, a Branson type B-220 ultrasonic bath (Danbury, CT) was used to extract the bile acids. The pellet was then rinsed with 0.1 N NaOH (2 ml) after centrifugation, and the supernatant was transferred to a glass test tube. A Waters Sep Pak tC18 cartridge (500 mg sorbent) primed with methanol (10 ml) and water (10 ml) was used to receive the combined extract. Water (5 ml), 15% acetone (4 ml), and water (5 ml) were all used to wash the cartridge in turn. Retained bile acids were evaporated to dryness under a N2 stream at a temperature below 40°C after being eluted with methanol (6 ml).

Method B: with alkaline hydrolysis step.

Isopropanol (500 µl) and 1 N NaOH (100 ml) were added after the cholylglycine hydrolase/sulfatase treatment stage (see procedure A), and the solution was incubated at 60°C for 2.5 h. The same as before, an IS, 50 nmol of norDCA, and 3 ml of 0.1 N NaOH were added, and the bile acids were extracted (17).

Derivatization of bile acids

Extracted unconjugated bile acids (either by method A or B) were derivatized to their 24-phenacyl esters (16) as follows: to the dried extract, 10 mg/ml of TEA in acetone (150 µl) and 12 mg/ml of phenacyl bromide (2-acetobromophenone) in acetone (150 µl) were added, and the mixture was heated at 50°C with ultra-sonication in a screw-capped glass tube for 1.5 h. The reaction mixture was diluted with acetone (2 ml) and applied to a Waters Sep-Pak® silica cartridge (500 mg sorbent), which had been primed with acetone (5 ml). To elute the bile acid 24-phenacyl esters completely, the

column was eluted with acetone (4 ml), and all the collected effluent was dried under an N2 stream.

Gradient Program:

The Gradient programme for Trial 2 of Simultaneous Bile Acid Estimation with Post Column Derivatization is shown in Table (b).

Trial 3

Reagents, chemicals, filters and column

Reagents, chemicals, filters, and column for Simultaneous Estimation of Bile Acid with Post Column Derivatization by Using HPLC are shown in Table (c).

Chromatographic conditions:

Instrument	:	HPLC
Column	:	YMC Pack ODS-AM, 150 x 4.6 mm, 3µm
Injection Volume	:	50 µL
Flow rate	:	1.50 mL/min
Wavelength	:	UV 205 nm
Column Temperature	:	45°C
Sample Temperature	:	25°C
Run Time	:	75 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.

Preparation of solutions

Dilute Orthophosphoric acid for buffer pH adjustment preparation:

20 mL of water and 10 mL of Orthophosphoric acid should be added to a 100 mL volumetric flask and well mixed. Mix after cooling and diluting with water to volume (18).

Buffer solution pH 2.5:

3.45 g of sodium dihydrogen phosphate monohydrate should be dissolved before being added to 1000 mL of Mill-Q water. By adding diluted phosphoric acid while stirring, the pH is adjusted to 2.5 +/- 0.05. Stop stirring, wait 5 to 10 minutes, and then check the pH. If necessary, adjust the pH. Pass through a 0.22 µm PVDF membrane filter to filter (19).

Mobile phase A:

Use 100% Buffer solution pH 2.5 as Mobile Phase A

Mobile phase B:

Use 100% Acetonitrile as Mobile Phase B

Gradient Program:

The Gradient programme for Trial 3 of Simultaneous Bile Acid Estimation with Post Column Derivatization is shown in Table (d).

Trial 4

Reagents, chemicals, filters and column for trial 4

Reagents, chemicals, filters, and column for HPLC-based simultaneous estimation of bile acid with post-column derivatization are shown in Table (e).

Chromatographic conditions:

Instrument	:	HPLC
Column	:	ACE3 Excel C18 250 x 4.6 mm, 3µm
Injection Volume	:	50 µL
Flow rate	:	0.80 mL/min
Wavelength	:	UV 205 nm
Column Temperature	:	45°C
Sample Temperature	:	25°C
Run Time	:	75 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.

Preparation of solutions

Dilute Orthophosphoric acid for buffer pH adjustment preparation:

Transfer 5.0 mL of Orthophosphoric acid and thoroughly combine it in a 50 mL volumetric flask with around 10 mL of water. Mix after cooling and diluting with water to volume.

Buffer solution pH 2.5:

6.903 g of sodium dihydrogen phosphate monohydrate should be dissolved before being added to 1000 mL of Mill-Q water. With diluted phosphoric acid while swirling, raise pH to 2.498. Stop stirring, wait 5 to 10 minutes, and then check the pH. If necessary, adjust the pH. Pass through a 0.45 µm PVDF membrane filter to filter (20).

Mobile phase A:

Use 100% Buffer solution pH 2.498 as Mobile Phase A

Mobile phase B:

Use 100% Acetonitrile as Mobile Phase B

Gradient Program:

The Gradient programme for Trial 4's Simultaneous Estimation of Bile Acid with Post Column Derivatization is shown in Table (f).

Result and discussion

Trial 1:

Observation:

- Only four peaks, namely cholic acid (CA), taurocholic acid (TA), Taurochenodeoxycholic acid (TCDA), and Glycocholic acid (GA), were found in the bile acid solution.
- Three further peaks were not seen in this chromatogram (Figure b).

Conclusion

- Improved gradient analysis to evaluate additional peaks and changes to sample preparation, such as post-column derivitization.

Trial 2

Observation

- Only five peaks, namely cholic acid (CA), taurocholic acid (TA), taurochenodeoxycholic acid (TCDA), glycocholic acid (GA), and glycochenodeoxycholic acid (GDA), were seen in the bile acid solution.
- Deoxycholic acid (DA) and chenodeoxycholic acids (CDA) had 2

additional peaks that this approach was unable to elute (figure c)

Conclusion

➢ To evaluate the remaining peaks, deoxycholic acid (DA) and chenodeoxycholic acids (CDA), the gradient programme has to be changed.

➢ Changing the gradient programme is necessary to enhance the peak form.

Trial 3

Observation

➢ Resolution between Cholic acid (CA), Taurocholic acid (TA) impurity peak found about 2.0 (Figure d).

➢ 6 Bile Acids peaks also separate from each other.

➢ No blank and Placebo interference at retention time of main peak and 6 Bile Acids peaks.

Conclusion

➢ Need to Change Gradient programm to eluate remaining peaks i.e. chenodeoxycholic acids (CDA).

➢ Need to Change Gradient programm to improve the peak shape.

Trial 4 (Final):

Observation-

- All Bile acids peaks are well separate from each other and main peak.
- No blank and placebo interference at the retention time of main peak and impurities peak (Figure e).

Conclusion-

From observation this methodology can be finalized and Prevalidation study needs to be performed (Table g).

Figures and Tables

Figures

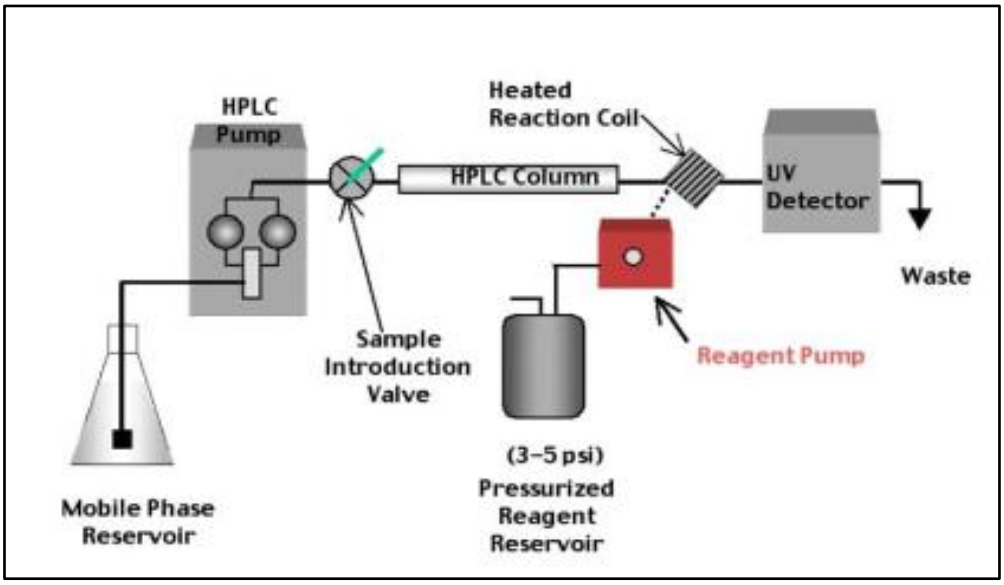

Figure (a) Post-column derivatization.

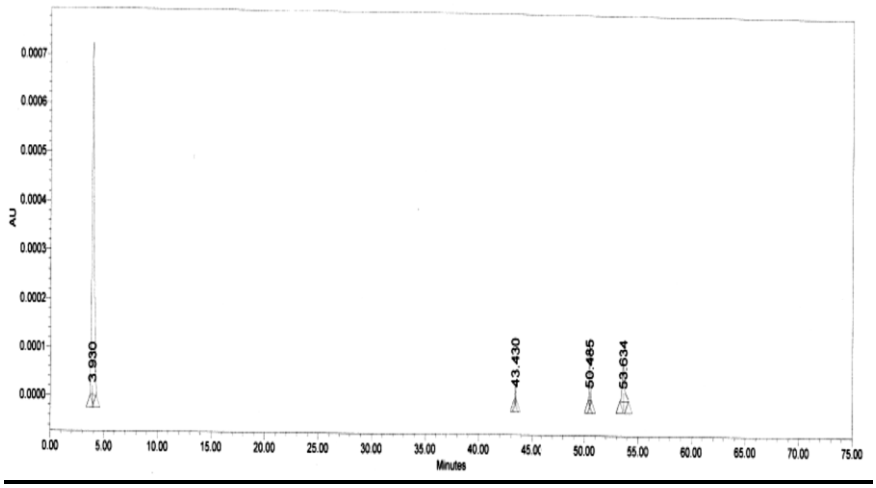

Figure (b) Chromatogram for trial 1 in Bile Acids (Postcolumn Derivatization)

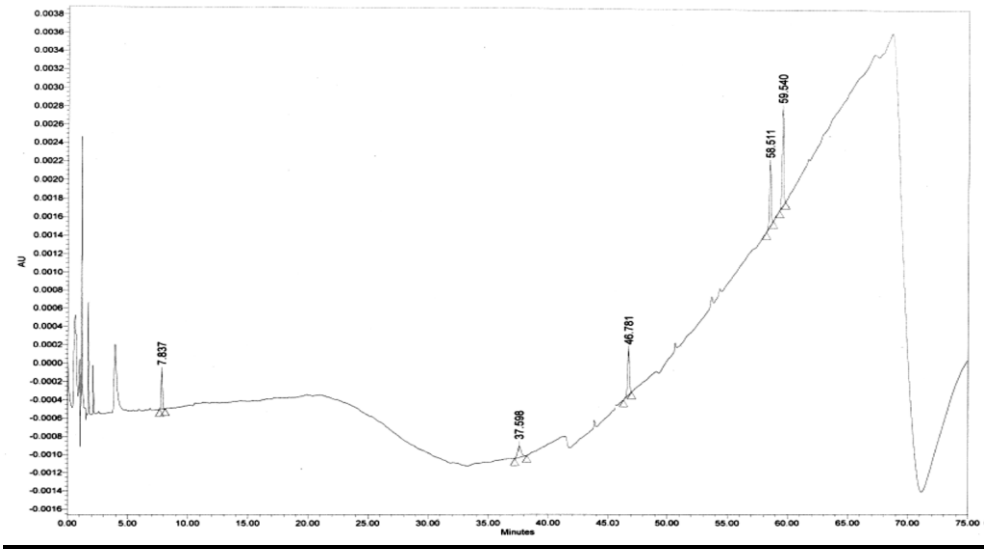

Figure (c) Chromatogram for trial 2 in Bile Acids (Postcolumn Derivatization)

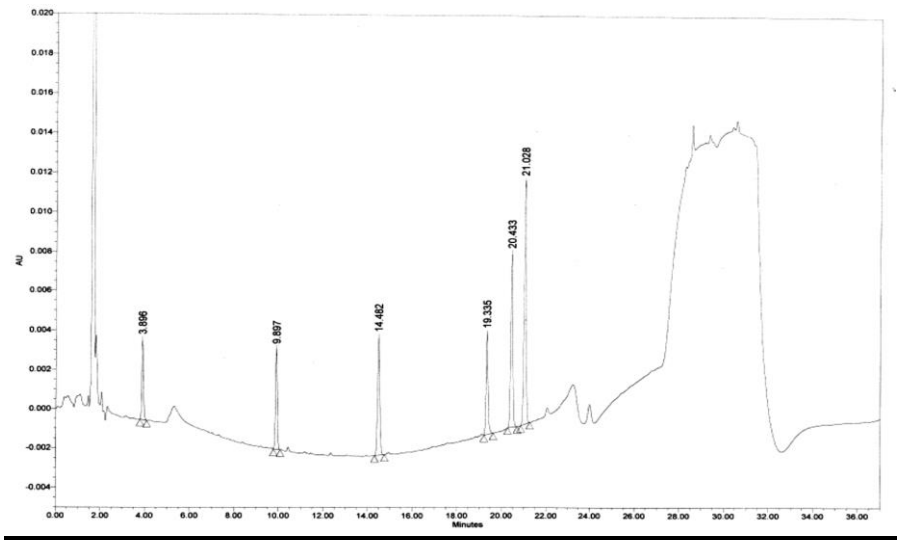

Figure (d) Chromatogram for trial 3 in Bile Acids (Postcolumn Derivatization)

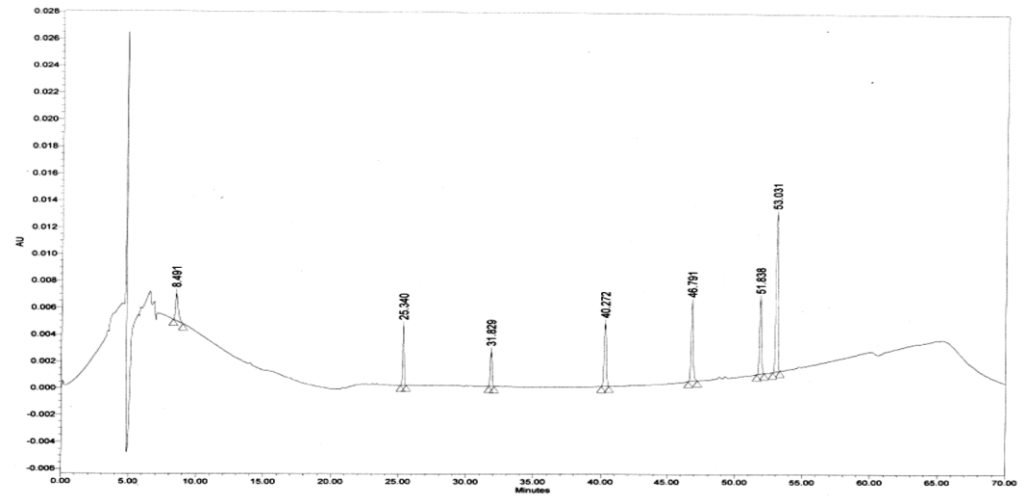

Figure (e) Chromatogram for trial 4 in Bile Acids (Postcolumn Derivatization)

Tables

Table (a) Reagents, chemicals, filters and column for bile acids (Postcolumn Derivatization)

Sr. No.	**Reagents, chemicals, filters and column**	**Grade**	**Make**
1.	Ortho-phosphoric acid (88%)	HPLC	Honeywell
2.	Sodium dihydrogen phosphate monohydrate	EMPARTA®	Merck
3.	Acetonitrile	Supra-Gradient	Bisolve
4.	0.22 µm PVDF membrane filter (Item No.HVLP04700)	--	Millipore
5.	0.45µ Nylon Syringe filter (Cat No. SYNN0602MNXX104)	--	Mdi
6.	YMC Pack ODS-AM, 150 x 4.6 mm, 3µm (P/N –AM12S03-1546WT)	--	YMC Pack

Table (b) Gradient program for Trial 2 in Bile acids (Postcolumn Derivatization)

Time (min.)	**Mobile Phase A (%)**	**Mobile Phase B (%)**
0	90	10
7	87	13
20	80	20
46	58	42
50	45	55
60	90	10
75	90	10

Table (c) Reagents, chemicals, filters and column in Trial 3

Sr. No.	**Reagents, chemicals, filters and column**	**Grade**	**Make**
1.	Ortho-phosphoric acid (88%)	HPLC	Honeywell
2.	Sodium dihydrogen phosphate monohydrate	EMPARTA®	Merck
3.	Acetonitrile	Supra-Gradient	Bisolve
4.	0.22 µm PVDF membrane filter (Item No.HVLP04700)	--	Millipore
5.	0.45µ Nylon Syringe filter (Cat No. SYNN0602MNXX104)	--	Mdi
6.	YMC Pack ODS-AM, 150 x 4.6 mm, 3µm (P/N –AM12S03-1546WT)	--	YMC Pack

Table (d) Gradient program for Trial 3 in Bile acids (Postcolumn Derivatization)

Time (min.)	**Mobile Phase A (%)**	**Mobile Phase B (%)**
0	92	8
35	85	15
45	74	26
67	54	46
69	92	8
75	92	8

Table (e) Reagents, chemicals, filters and column for trial 4

Sr. No.	**Reagents, chemicals, filters and column**	**Grade**	**Make**
1.	Ortho-phosphoric acid (88%)	HPLC	Honeywell
2.	Sodium dihydrogen phosphate monohydrate	EMPARTA®	Merck
3.	Acetonitrile	Supra-Gradient	Bisolve
4.	0.22 µm PVDF membrane filter (Item No.HVLP04700)	--	Millipore
5.	0.45µ Nylon Syringe filter (Cat No. SYNN0602MNXX104)	--	Mdi
6.	YMC Pack ODS-AM, 150 x 4.6 mm, 3µm (P/N –AM12S03-1546WT)	--	YMC Pack

Table (f) Gradient program for Trial 4 in Bile acids (Postcolumn Derivatization)

Time (min.)	**Mobile Phase A (%)**	**Mobile Phase B (%)**	**Flow rate(mL/min)**
0	96	4	0.60
10	90	10	0.60
20	75	25	0.80
45	55	45	0.80
60	45	55	0.80
65	96	4	0.60
75	96	4	0.60

Table (g) Estimation of Bile Acid with Post Column Derivatization

Sr. No	**Peak Name of bile acid**	**Retention time (min)**	**Area (Uv sec)**	**% Area**	**Height (Uv)**	**USP Resolution**	**Asym**	**USP Tailing**	**USP Plate count**
1	Taurocholic acid (TA)	8.499	8524	7.47	658	28.30	4761	1.7	10848
2	Cholic acid (CA)	25.335	9985	8.75	1374	60.30	262717	1.1	277172
3	Taurochenodeoxycholc acid (TCDA)	31.822	7678	6.73	814	29.00	278797	1.0	258534
4	Glycocholic acid (GA)	40.267	60980	17.62	5840	30.80	307671	1.1	483697
5	Glycochenodeoxycholic acid (GDA)	46.773	55766	16.14	5565	22.29	475663	1.0	607415
6	Chenodeoxycholic acids (CDA)	51.118	120198	34.79	11835	18.60	596051	1.0	611116
7	Deoxycholic acid (DA)	53.014	59300	32.20	9266	4.4	621630	1.0	6012143

Conclusion

As a result of the renewed interest in bile acids over the past decade due to their use in the treatment of gallstones and the potential role they may play in the etiology of a variety of disorders, analytical methods have been enhanced. Particularly, advancements have been made towards more comprehensive assays, such as the determination of glucuronide conjugates, sulphate esters, and minor constituent bile acids in biological fluids, as well as the widespread adoption of solid extraction techniques and the enzymatic hydrolysis of conjugated bile acids. Detector systems that have not been adequately evaluated for use when bile acids are present in low concentrations impede the use of HPLC, despite its effectiveness as a separation technique. HPLC procedures are currently the most effective instruments for the precise and comprehensive examination of biological fluids, but they are time-consuming, expensive, and unsuitable for routine clinical use (21).

Acknowledgements

I would like to express my thankfulness to all the supporting persons who helped me

to prepare this article specially I am very grateful to my guide and the team of university for their cooperation and support.

References

1. Ye L., et al., (2007). High-performance liquid chromatography–tandem mass spectrometry for the analysis of bile acid profiles in serum of women with intrahepatic cholestasis of pregnancy, Journal of Chromatography B, 860, 10-17.
2. Corleto KA., Singh J., Jayaprakasha GK., Patil BS. (2019). A sensitive HPLC-FLD method combined with multivariate analysis for the determination of amino acids in l-citrulline rich vegetables, Journal of food and drug analysis, 27, 717-728
3. Furota S., et al., (2018). Quantitative analysis of underivatized amino acids in the sub to several-Nano molar range by ion-pair HPLC using a corona-charged aerosol detector (HPLC–CAD), Journal of Chromatography B, 1095, 191-197
4. Lamp A., et al., (2018). Improved HPLC-method for estimation and correction of amino acid losses during hydrolysis of unknown samples, Analytical Biochemistry, 543, 140-145
5. Dimova et al (2003).RP-HPLC Analysis of amino acid with UV detection, Academic bulgare of science tome 56, no, 12, 2003 75-78
6. Rasdin Ridwan, et al (2018), "Development of Isocratic RP-HPLC Method for Synopsis Shree JJT University, Rajasthan Vidyanagari-3330001 (23) Separation and Quantification of L-Citrulline and L-Arginine in Watermelons", IJAS vol. 2018, Article 9 pages, 2018.
7. Zinhong shi, et al (2013) Pre-column Derivatization RP-HPLC Determination of Amino Acids in Asparagi Radix before and after Heating Process5; 2013; 351-356.
8. Pravin Bhandare et al (2010) Determination of amino acid without derivatization by using HPLC - HILIC column J.CHEM.PHARM.RES .2(2) 372-380
9. Fabiani et al (2002) High-Performance Liquid Chromatographic Analysis of Free Amino Acids in Fruit Juices Using Derivatization with 9-Fluorenylmethyl- Chloroformate, February 2002 Journal of Chromatographic Science 40(1):14-8
10. Alahmad, Kamal et al (2016) Advanced Techniques of TLC, HPLC, GC, and Applications for Study and Analysis Amino Acids & Peptide Compounds 4; 6 1090- 1102.
11. Li, Y.W., Zhu, X.Y., But, P.P.H., Yeung, H.W.; Ethnopharmacology of bear gall bladder: I; Journal of Ethnopharmacology, (1995); 47: 27 – 31.
12. Li, T., Yang, Z., Cai, H.J., Song, L.W., Lu, K.Y., Zhou, Z., et al.; Effects of in vitro cultivated Calculus Bovis compound on pulmonary lesions in rabbits with schistosomiasis; World Journal of Gastroenterology, (2010); 16: 749–754.
13. Cai, H.J., Wang, S.Y., Zhang, Y.H., Li, Q.M., Xia, J.Y., Wang, Q., et al.; In vitro cultured Calculus Bovis in the treatment of epidemic encephalitis B; Acta Medicine Universities Scientiae et Technologiae Huazhong, (2003); 32: 604–606.
14. Liang, J.F., Hu, J.X., Yang, B.Y., Chen, S.Q., Zhong, Y., Liang, Y.J.; Artificial Calculus Bovis inhibits neuron loss in hippocampus and hilus and protects the GAD positive cells in hippocampus of epileptic rats; Chinese Journal of Pathophysiology, (2006); 22: 2414–2417.

15. Tai, C.S., Wang, Z.Y., Jia, G., Wang, T.C., Wang, X., Ma, W.J., et al.; Study on the protective effect of bezoar and its Chinese herbal preparation on the lipid peroxidation induced by n-hexane in livers and kidneys of mice; China Occupational Medicine, (2006); 33: 417–419.
16. Yan, S.K., Xin, W.F., Luo, G.A., Wang, Y.M., Cheng, Y.Y; An approach to develop two-dimensional fingerprint for the quality control of Qingkailing injection by high-performance liquid chromatography with diode array detection; Journal of Chromatography A, (2005); 1090: 90–97.
17. Jiang, P., Dou, S.S., Liu, L., Zhang, W.D., Chen, Z.L., Xu, R.L., et al.; Identification of multiple constituents in the TCM-formula Shexiang Baoxin pill by LC coupled with DAD-ESI-MS-MS; Chromatographia, (2009); 70: 133–142.
18. Takahashi, K., Azuma, Y., Shimada, K., Saito, T., Kawase, M., Schaffer, S.; Quality and safety issues related to traditional animal medicine: Role of taurine; Journal of Biomedical Science, (2010); 17: S44.
19. Takahashi, K., Azuma, Y., Azuma, J., Schaffer, S.W.; Taurine and Calculus Bovis: Benefits of Ethnopharmacological knowledge; Amino Acids, (2011); 41: S84.
20. Qin, X.F.; Bilirubin would be the indispensable component for some of the most important therapeutic effects of Calculus Bovis (Niuhuang); Chinese Medical Journal, (2008); 121: 480.
21. Wan, T.C., Cheng, F.Y., Liu, Y.T., Lin, L.C., Sakata, R; Study on bioactive compounds of in vitro cultured Calculus Suis and natural Calculus Bovis; Animal Science Journal, (2009); 80: 697–704.