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A Simple Dilute-and-Shoot Flow-Injection Mass Spectrometric Method for Quantification of Glycocholic Acid in Human Bile

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Abstract

Levels of glycocholic acid are elevated in the bile of cholangiocarcinoma patients, making it a potential biomarker for the disease. The utility of liquid chromatography mass spectrometry based methods for the quantification of glycocholic acid often suffers from time-consuming extraction steps, carryover, and unavailability of blank bile matrix. To overcome the problems, we developed a dilute-and-shoot flow-injection tandem mass spectrometry (standard addition-based) method for quantification of glycocholic acid in bile. Bile was first diluted, followed by spiking the glycocholic acid standard and internal standard into the diluted bile, which was then directly flow-injected (without chromatographic separation) into the electrospray ionization source. Detection was carried out in negative multiple reaction monitoring mode with m/z transitions set at 464.1→74.0 and 401.2→249.1 for glycocholic acid and internal standard, respectively. A standard addition calibration curve was constructed to determine the amount of glycocholic acid present in human bile. The method has been validated with clinical samples according to FDA guidelines. Linearity was achieved in the spiked concentration range of 12.5 to 200 ng/mL with a correlation coefficient of 0.9857. The method has good accuracy, precision, minimal matrix effect, and is sufficiently sensitive enough to quantitate glycocholic acid even after 800,000-fold dilution. In conclusion, a dilute and shoot flow-injection MS/MS method has been developed and validated for the first time, which could be used in routine analysis of glycocholic acid in human bile in a clinical setting.

Keywords: Glycocholic acid; FI-MS/MS; Bile; Standard-addition; Validation; Clinical

Introduction

Cholangiocarcinoma (CCA) or bile duct cancer arises from the epithelial lining of intra-hepatic and extra-hepatic bile ducts. CCA is one of the most aggressive forms of malignancies and around 8000 Americans are diagnosed annually [1-3]. Early diagnosis of CCA is challenging due to its late clinical presentation and difficulties in early detection. Current diagnostic methods lack the ability to distinguish benign from malignant tumors. For example, imaging techniques such as ultrasonography, computed tomography (CT), and magnetic resonance imaging (MRI) reveal bile duct blockage, but they are inefficient in differentiating the blockage caused by benign scarring or a malignant tumor [1].

Glycocholic acid (GCA) is a conjugated bile acid produced from cholic acid. A significant increase in the concentration of GCA has been observed in the bile of patients with CCA, compared to those with benign biliary tract diseases [3,4]. Because bile and its constituents are directly in contact with the biliary epithelium, it is an ideal biological fluid for detection of CCA, however it was not until recently a preferred medium because sampling of bile required surgery [4]. Advances in imaging techniques like endoscopic retrograde cholangiopancreatography now makes it possible to sample bile without surgical intervention.

Currently, LC-MS is the method of choice for quantifying GCA in bile [5]. Although LC-MS has unparalleled sensitivity and accuracy, it suffers from various obstacles due to the highly hydrophobic nature of bile acids and matrix components that complicate its quantification. First, current methods require time-consuming extraction steps in order to separate GCA from interfering matrix components. Further, because bile acids bind to the C18 column strongly, it is necessary to frequently wash the column vigorously, which is not practical in a clinical setting. Finally, it is virtually impossible to create a blank bile matrix that does not contain GCA.

To overcome these problems, we employed dilute-and-shoot flow-injection MS/MS (for short, FI-MS/MS) to quantify GCA in bile, for which standard addition calibration is employed. The high concentration of GCA in bile allows dilution of bile by as much as 800,000 times to minimize the matrix effect [6]. Diluted sample is then injected directly into the ion source without LC separation, avoiding column contamination and carryover. In addition, sample preparation and run time are substantially minimized by a FI-MS/MS method, whilst maintaining both quantification accuracy and precision [7,8]. Moreover, we utilized a standard-addition method for calibration, which not only does not require a blank matrix that is virtually impossible to obtain, but also minimizes matrix effects. In standard addition, solutions containing different concentrations of analyte are directly added to aliquots of the sample [9]. Thus, any change in signal intensity between the sample and the spiked samples will only result from change in analyte concentration and hence matrix effects are minimized. Bio analytical method validation has been conducted in accordance with FDA guidelines [10].

Materials and Methods

Chemicals

Glycocholic acid and internal standard (dehydrocholic acid) were purchased from Sigma Aldrich, (St. Louis, MO, USA). HPLC grade methanol was purchased from EMD Millipore Corporation (Billerica, MA, USA). Deionized water was obtained from Barnstead D3750 Nano Pure water purification system by Thermo Scientific (Waltham, MA, USA). Bile samples were obtained from four volunteers at Mississippi State University.

Instrumentation

Quantification was performed by liquid chromatography coupled to a tandem mass spectrometer. The Shimadzu HPLC unit (Columbia, MD, USA) consisted of binary pumps (Nexera LC-30 AD), degasser (a DUG20A3R), autosampler (SIL-30 AC), column oven (CTO-10AVP) and a controller (CBM 20A). The AB SCIEX 5500 QTRAP mass spectrometer (Toronto, Canada) was equipped with an electrospray ionization probe and a syringe pump. The instrument operation, acquisition and processing of data was performed using AB SCIEX Analyst software.

Flow injection

Flow injection of GCA and IS was performed isocratically at 30°C with a carrier solvent comprising of 90% methanol at a flow rate of 0.3 mL/min. It should be noted that a LC column was not used for separation. A 10 µL volume of the sample from the autosampler vials was injected into the system and data was acquired for a run time of 2.5 min per sample.

Tandem mass spectrometry

Mass spectrometric analysis was performed in negative electrospray ionization (ESI-) mode. Source and compound dependent parameters were optimized and the conditions were as follows: curtain gas: 30 psi; ion spray voltage: -4500 V; ion spray temperature: 400°C; ion source gases: 1 and 2: 20 psi; declustering potential: -110 V; entrance potential: -14 V; collision energy: -53 eV and cell exit potential: -25 eV. Quantitation of GCA and IS was achieved in multiple reaction monitoring (MRM) mode. The selected precursor-product ion pairs for GCA were m/z 464.1→74.0 and m/z 401.2→249.1 for IS, respectively.

Preparation of stock and working solutions

Stock solutions of GCA and IS were prepared separately in appropriate volumes of methanol to obtain concentrations of 1 mg/mL. Both stock solutions were stored at -20°C. The working standard solutions of GCA (2.5, 5, 10, 20, 30, and 40 µg/mL for calibrators and 6.3, 14.1, 32 µg/mL for quality controls (QCs)) were prepared by serial dilution in methanol. A working standard solution of IS (20 µg/mL) was prepared by diluting the stock solution of IS with methanol.

Preparation of bile samples by dilution

Bile samples were thawed to room temperature and vortexed for 2 minutes. 2 µL of each bile sample was diluted to 8 mL with methanol, vortexed for 2 min and centrifuged at 14000 rpm for 20 minutes. A pooled bile sample (mixture of 4 lots of human bile samples) was also treated in a similar way to yield diluted pooled bile. The supernatants obtained after centrifugation were used to prepare calibrators and quality controls as described below.

Preparation of bile calibrators for standard addition

Bile GCA calibrators (12.5, 25, 50, 100, 150 and 200 ng/mL) were prepared by spiking each working standard solution (2.5, 5, 10, 20, 30 and 40 µg/mL) with 5 µL of diluted pooled human bile. A single blank was prepared without spiking the diluted pooled bile with working standard solution. To ensure that the analyte concentration in the sample was within the linear range and not below the LLOQ, an 'initial spike' of 5 µL of 5 µg/mL of GCA was spiked into all the calibrators. The volume of the solutions was then made up with methanol, vortexed, centrifuged at 14000 rpm for 20 min and the supernatant was transferred to an auto sampler vial for analysis.

Preparation of quality controls (QCs)

GCA quality controls (QCs) (31.3, 70.7 and 160 ng/mL) were prepared by spiking working standard solutions (6.3, 14.1 and 32 µg/mL) with 5 µL of diluted pooled human bile and treated the same way as calibrators.

Method validation

The method was validated with human bile for linearity, accuracy, precision, lower limit of quantitation, matrix effect and carryover according to the FDA guidelines for bio analytical method validation.

Results and Discussion

Method development

Glycocholic acid has a molecular weight of 464.624 g/mol. Dehydrocholic acid was selected as internal standard not only because of its structural similarity to GCA, but also because it was significantly less expensive than the deuterated analogs of GCA. The IS has a molar mass of 402.531 g/mol.

MRM

Detection of GCA and IS was carried out using a triple quadrupole mass spectrometer. Since both GCA and IS are weak acids, negative ion mode was chosen for ionization. MRM was utilized to ensure specific quantification by reducing the interference of other molecules present in the bile. Experimentally, a precursor ion was first selected, followed by collision-induced dissociation after which a fragmentation ion of the precursor ion was selected for quantification. The precursor and fragment ions selected for this study are shown in Figure 1, where the peaks at 464.1 and 401.2 represent the precursor ions and peaks at 74.0 and 249.1 represent the product ions for GCA and IS, respectively. Specifically, the transitions used in MRM were 464.1→74.0 for GCA and 401.2→249.1 for IS. To ensure that only GCA and no other ions were quantified, we also monitored an additional transition of 464.1→402.4 as a qualitative transition for increased confidence in identifying the target analyte.

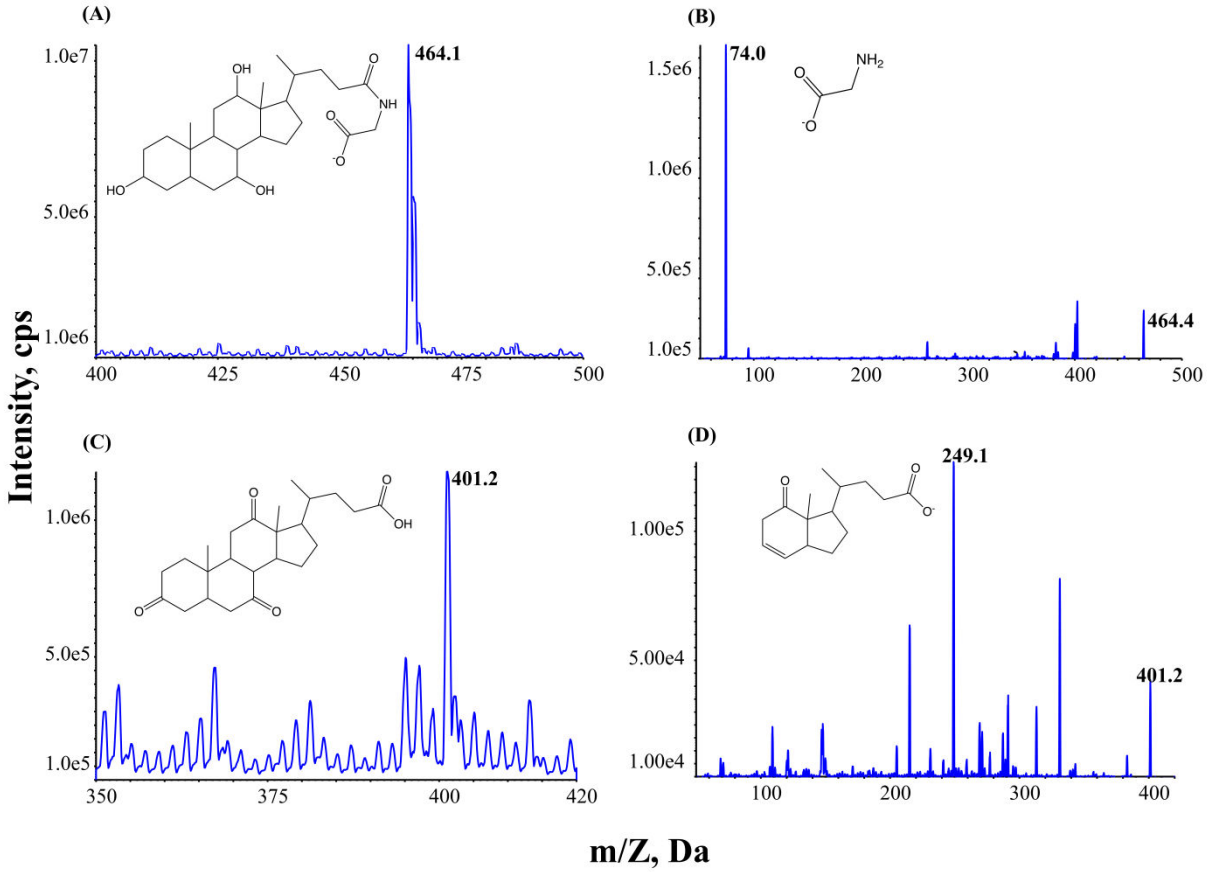

Figure 1: The mass spectra of (A) precursor ion of GCA (B) product ion of GCA (C) precursor ion of IS (D) product ion of IS in negative electrospray ionization mode.

Sample preparation and flow injection

Because both GCA and IS are highly hydrophobic, they can bind to C18 materials very strongly, resulting in carry over, which poses a problem when a column is used for separation of bile acid molecules. Hence, we employed a flow injection strategy to avoid this issue by eliminating the use of a column. This was possible considering that the concentration of GCA in bile is very high, meaning that GCA can still be detected even after substantial dilution of bile, and most importantly, such simple dilution may be sufficient enough to avoid the matrix effect when the separation column is not used. Clearly, another benefit of dilution is that no extraction method is needed to first retrieve GCA from bile before analysis. Hence, the bile is substantially diluted and then directly injected (without any sample preparation) into the mass spectrometer. A carrier solvent comprising of 90% methanol carried the injected sample as a plug into ESI of mass spectrometer at a flow rate of 0.3 mL/min. We found that our method was reproducible, evidenced by the fact that the detection signals were consistently detected at the same time window in every run. The typical flow injection detection signals of GCA and IS were displayed in Figure 2.

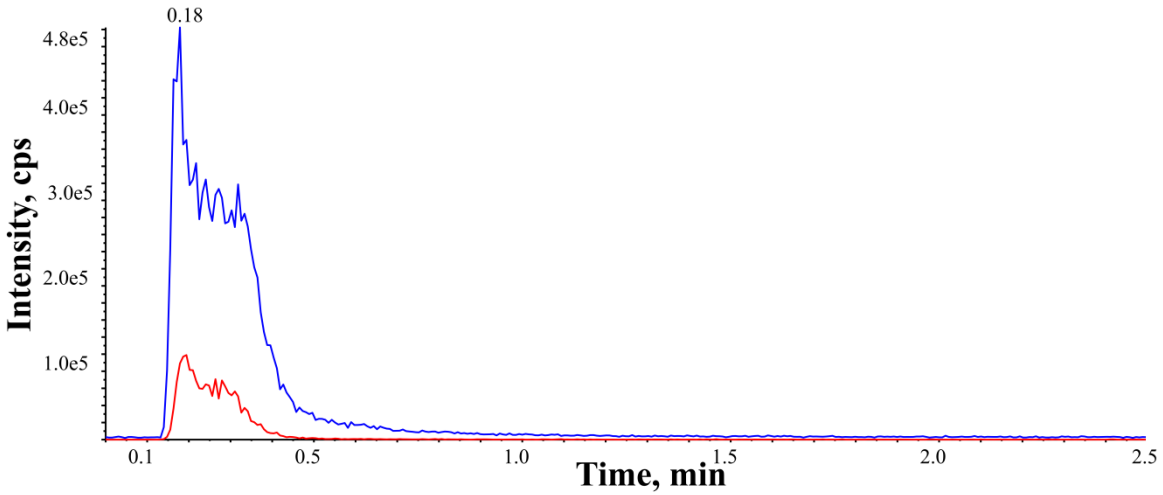

Figure 2: A typical FI-MS/MS chromatogram of GCA (red) and IS (blue) in human bile.

Standard addition

To quantify GCA in bile using a traditional calibration curve method, a blank bile matrix without GCA is required. However, it is virtually impossible to obtain such blank matrix, as the charcoal stripping method currently used not only removes GCA from bile, but also removes other matrix components. As a consequence, the actual composition of matrix after charcoal stripping is altered, not reflecting the true nature of the bile sample [11]. Standard addition is the method of choice for quantitation when the endogenous amount of analyte is "inherently" present in matrix [9,12,13]. In our method, we employed standard addition to generate the calibration curve, which was constructed using bile samples spiked with standard GCA solutions by plotting the peak area ratio of GCA to IS vs the concentration of GCA added. The absolute value of the x-axis intercept corresponds to the concentration of GCA in the bile sample. As illustrated in Figure 3, standard solutions containing different concentrations of GCA were directly added to aliquots of a bile sample.

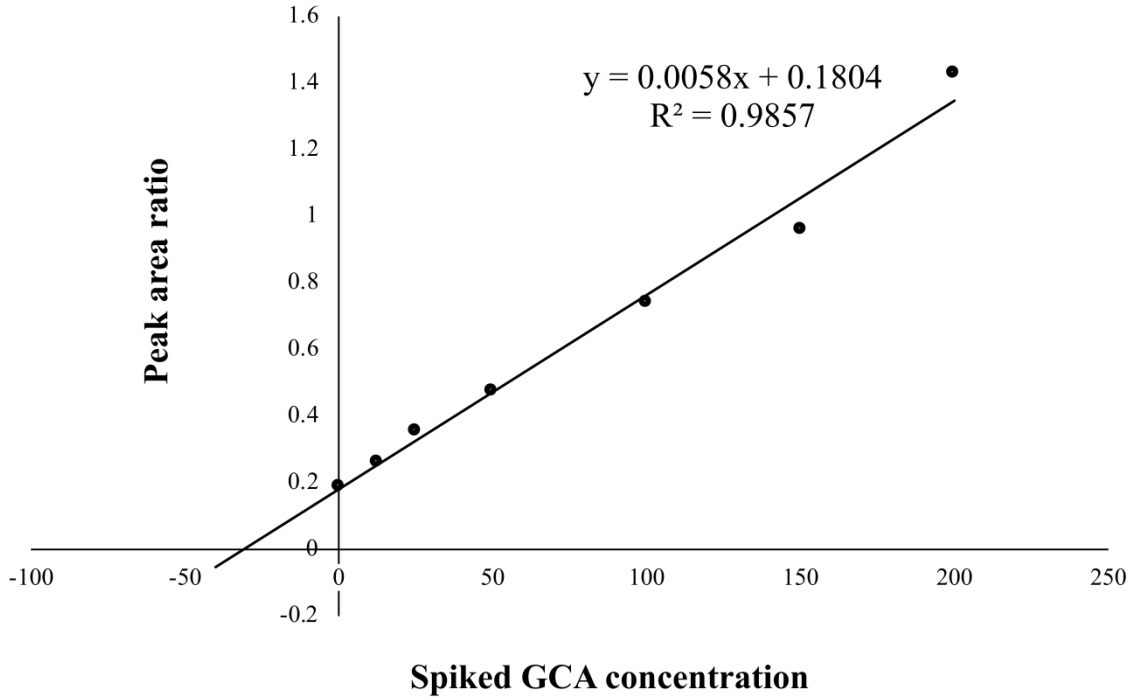

Figure 3: Standard-addition calibration plot of GCA in pooled human bile from four lots. The x-axis intercept corresponds to the actual concentration of GCA in the sample and can be obtained from the equation, y=0.0058x+0.1804. Correlation coefficient=0.9857.

Method validation

The method was validated for accuracy, precision, matrix effects, LLOQ and carryover according to the FDA Bio analytical Method guidelines.

Linearity of standard addition calibration curve

A linear relationship between the concentration of GCA and the peak area ratio (the fragment ion intensity of GCA to the fragment ion intensity of IS) was determined using pooled and individual human bile lots by plotting standard addition calibration curves. To evaluate linearity, five replicates of spiked GCA bile calibrators at 12.5, 25, 50, 100, 150, 200 ng/mL and single blank were analyzed. The chromatograms are displayed in Figure 4. Standard addition-calibration curves were plotted for pooled human bile and individual lots with peak area ratios vs concentration using weighing factor of 1/x. Linearity was found to be good with a correlation coefficient of 0.9857 as shown in Figure 3. The standard deviation and % RE values are presented in Table 1.

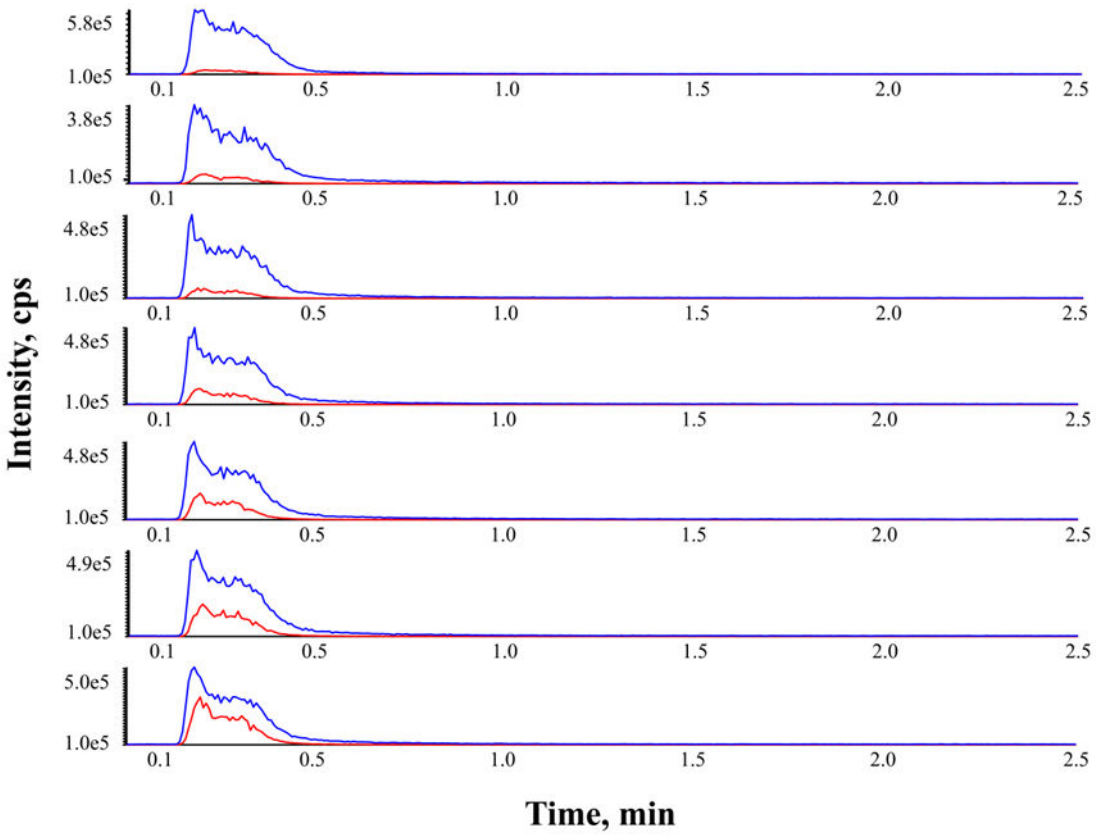

Figure 4: Linearity: FI-MS/MS chromatograms of calibrators in human bile (top to bottom) Single blank, 12.5 ng/mL, 25 ng/mL, 50 ng/mL, 100 ng/mL, 150 ng/mL and 200 ng/mL of spiked GCA.

Spiked concentration (ng/mL)	Determined concentration (ng/mL)	%RE	%CV
12.5	12.04 ± 2.45	-3.7	20.37
25	26.73 ± 5.26	6.9	19.66
50	50.95 ± 5.80	1.9	11.39
100	97.63 ± 4.08	-2.4	4.18
150	133.17 ± 6.90	-11.2	5.18
200	217.17 ± 10.60	8.6	4.88

Table 1: Linearity data of calibrators in pooled bile samples (n=3) obtained from six non zero calibrators using three lots of pooled bile. %RE (percent relative error)=[(determined concentration-spiked concentration)/(spiked concentration)] × 100. %CV (percent coefficient of variation)=(standard deviation/mean) × 100.

Accuracy and precision

The accuracy of a method defines the closeness of mean test concentration determined by the method to the actual concentration of the analyte, while precision of a method defines the closeness of individual concentrations of analyte to one another. Accuracy and precision of our method were evaluated by analyzing five replicates of QCs at 31.25, 70.71 and 160 ng/mL on the same day (intra-assay) and five separate days (inter-assay). The data for intra-assay and inter-assay

accuracy and precision were presented in Tables 2 and 3. Accuracy, expressed as % RE of intra-assay for five replicates on same day was -1.44-18.1 while % RE of inter-assay for five replicates on five separate days was 9.52-14.8. Precision, expressed as % CV of intra-assay for five replicates on same day was 11.45-12.53 while % CV of inter-assay for five replicates on five separate days was 6.50-11.60. This study clearly suggests that our method is highly reproducible.

	Spiked concentration (ng/mL)	Determined concentration ± SD (ng/mL)	%RE	%CV
LQC	31.25	33.08 ± 6.56	5.86	12.53
MQC	70.71	83.51 ± 9.73	18.1	11.45
HQC	160.00	157.7 ± 16.70	-1.44	12.34

Table 2: Intra-assay accuracy and precision of GCA in pooled bile (n=5) were determined at three QC levels using five lots of pooled bile on same day. %RE (percent relative error)=[(determined concentration - spiked concentration)/(spiked concentration)] ×100. %CV (percent coefficient of variation)=(standard deviation/mean) × 100.

	Spiked concentration (ng/mL)	Determined concentration ± SD	%RE	%CV
LQC	31.25	33.57 ± 6.2	14.80	9.02
MQC	70.71	69.61 ± 31.28	9.52	11.60
HQC	160.00	141.8 ± 18.38	12.03	6.50

Table 3: Inter-assay accuracy and precision of GCA in pooled bile (n=5) were determined at three QC levels using five lots of pooled bile on five different days. %RE (percent relative error)=[(determined concentration-spiked concentration)/ (spiked concentration)] × 100. %CV (percent coefficient of variation)=(standard deviation/mean) × 100.

Matrix effect

The presence of other molecules in a sample can lead to matrix effects (i.e., ion suppression or enhancement) and bile does contain high concentrations of various other molecules. Hence, both absolute and relative matrix effects on our method were determined by comparing the peak area ratios of five replicates of QCs in bile samples and solvent. Absolute matrix effects were determined by comparing the peak area of GCA in diluted bile samples and standard solutions in solvent at three QCs. Relative matrix effects were determined by comparing the peak area ratios of GCA/IS in diluted bile samples and standard solutions in neat solution at three QCs. The data is presented in Table 4. The percentage absolute matrix effect expressed as % ME was 91.65-98.99 while relative matrix effect expressed as % ME was 87.35-94.78. The data suggest that the use of dilution and standard addition held the matrix effect low and within the 15% limit set by the FDA guidelines.

	Spiked concentration (ng/mL)	Absolute matrix effect ± SD	%CV	Relative matrix effect ± SD	%CV
LQC	31.25	98.99 ± 10.37	10.47	94.78 ± 45	9.97
MQC	70.71	96.36 ± 5.32	5.53	87.35 ± 3.54	4.05
HQC	160.00	91.65 ± 4.87	5.31	91.66 ± 7.90	8.62

Table 4: Absolute and relative matrix effects of GCA in pooled bile (n=5) were determined at three QC levels using five lots of pooled human bile. Absolute matrix effect=(mean peak area of GCA in diluted bile)/(mean peak area of GCA in neat solution). Relative matrix effect=(mean peak area ratio of GCA/IS in diluted bile)/(mean peak area ratio of GCA/IS in neat solution).

Lower limit of quantitation

In our method, the lower limit of quantitation (LLOQ) was defined as the concentration of GCA in the lowest calibrator (12.5 ng/mL) used to construct the standard addition calibration curve. We have conducted a study to examine reproducibility of quantitation at the LLOQ level of 12.5 ng/mL, which was achieved by quantifying GCA in six replications of a pooled bile samples containing 12.5 ng/mL. It was found that the % CV was 11%, suggesting that our method is sensitive enough to reproducibly detect GCA at a concentration as low as 12.5 ng/mL. It must be noted that no study was conducted to determine whether our method can detect GCA at the level of less than 12.5 ng/mL.

Carry over

Carry over problem is often associated with the column-based method, where the analyte from earlier analyzed samples can stick to the column, affecting analysis of the samples that are analyzed thereafter. This is particularly problematic with bile that contains high concentrations of various highly hydrophobic molecules including GCA. Although our method does not use any column, we did conduct a study to examine whether any bile molecules including GCA can stick to auto sampler and tubing, leading to carry over, and thus affecting the accuracy of our method. This was evaluated by immediately quantifying a blank sample after injecting GCA of 200 ng/mL (upper limit of quantitation). It was found that our method was not subjected to the carry over problem as expected.

Quantification of GCA in human bile samples

After developing and validating our method, we applied it to quantify the level of GCA in human bile samples collected from four individuals. It was found that our method could detect and differentiate the GCA and IS signals from the background noise. The standard addition curves were constructed for each of all the four bile samples. The result obtained from four human bile samples was listed in Table 5.

Human bile sample	Concentration of GCA (mM)
Subject 1	4.53
Subject 2	24.95
Subject 3	9.96
Subject 4	21.48

Table 5: Concentration of GCA in four different human bile samples obtained from standard addition plots. All values are expressed in mM.

Perwaiz et al. reported that the GCA levels in six subjects determined using their LCMS method were between 5.04-55.04 mM [5]. The values obtained from our method were within the same range further demonstrating that our method is reliable for quantification.

Conclusion

We have successfully developed a dilute-and-shoot flow injection MS/MS method for the quantification of GCA in human bile using standard addition. Our method has been validated for linearity, accuracy, precision, matrix effects, LLOQ and carryover according to the FDA guidelines. This method offers several benefits for clinical use. First, it does not require tedious sample purification prior to analysis. Our method utilizes simple dilution for sample preparation, while not compromising on sensitivity even after 800, 000-fold dilution. Second, our method is accurate and precise without the use of LC separation, eliminating the carryover problem that which would otherwise require frequent washing to maintain the column condition. Third, our method does not necessitate a blank bile matrix that is virtually impossible to obtain but is required for the conventional quantitation method. In addition, our method is fast with a run time of only 2.5 min, enabling high throughput analyses of over 500 samples a day. To the best of our knowledge, this is the first flow injection MS/MS method developed for quantifying GCA in human bile.

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References

1. Janvilisri T, Leelawat K, Roytrakul S, Paemanee A, Tohtong R (2015) Novel Serum Biomarkers to Differentiate Cholangiocarcinoma from Benign Biliary Tract Diseases Using a Proteomic Approach.
2. https://www.cancer.org/cancer/bile-duct-cancer/about/key-statistics.html
3. Xu X, Cheng S, Ding C, Lv Z, Chen D, et al. (2015) Identification of bile biomarkers of biliary tract cancer through a liquid chromatography/mass spectrometry-based metabolomic method. Mol Med Rep 11: 2191-2198.
4. Sharif AW, Williams HR, Lampejo T, Khan SA, Bansi DS, et al. (2010) Metabolic profiling of bile in cholangiocarcinoma using in vitro magnetic resonance spectroscopy. HPB (Oxford) 12: 396-402.
5. Perwaiz S, Tuchweber B, Mignault D, Gilat T, Yousef IM (2001) Determination of bile acids in biological fluids by liquid chromatography-electrospray tandem mass spectrometry. J Lipid Res 42: 114-119.
6. Stahnke H, Kittlaus S, Kempe G, Alder L (2012) Reduction of matrix effects in liquid chromatography-electrospray ionization-mass spectrometry by dilution of the sample extracts: how much dilution is needed? Anal Chem 84: 1474-1482.
7. Nanita SC, Kaldon LG (2016) Emerging flow injection mass spectrometry methods for high-throughput quantitative analysis. Anal Bioanal Chem 408: 23-33.
8. Alagandula R, Zhou X, Guo B (2017) A dilute-and-shoot flow-injection tandem mass spectrometry method for quantification of phenobarbital in urine. Rapid Commun Mass Spectrom 31: 39-46.
9. Cai X, Liu Y, Zhou X, Navaneethan U, Shen B, et al. (2012) An LC-ESI-MS method for the quantitative analysis of bile acids composition in fecal materials. Biomed Chromatogr 26: 101-108.
10. https://www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf
11. Griffiths WJ, Sjövall J (2010) Bile acids: analysis in biological fluids and tissues. J Lipid Res 51: 23-41.
12. Ito S, Tsukada K (2002) Matrix effect and correction by standard addition in quantitative liquid chromatographic-mass spectrometric analysis of diarrhetic shellfish poisoning toxins. J Chromatogr A 943: 39-46.
13. Basilicata P, Miraglia N, Pieri M, Acampora A, Soleo L, et al. (2005) Application of the standard addition approach for the quantification of urinary benzene. J Chromatogr B Analyt Technol Biomed Life Sci 818: 293-299.