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Method for the quantitative assay of fatty acid–bile acid conjugates by tandem mass spectrometry

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Abstract

Fatty acid–bile acid conjugates and especially arachidyl amido cholic acid are synthetic molecules that were shown to prevent cholesterol gallstone formation in mice and hamsters as well as to dissolve pre-existing gallstones in mice. To measure these novel compounds we developed a liquid chromatography electrospray tandem mass spectrometry method based on the analysis of 100 μl of plasma with stearyl amido cholic acid (stamchol, 1.5 μmol/l) added as internal standard. Repeatable calibrations between 0 and 50 μmol/l exhibited consistent linearity and reproducibility. Inter- and intraassay C.V.s were 5.3–11.4% and 2.6–6.4%, respectively, at targeted concentrations of 0.1, 2.3 and 50 μmol/l.

Keywords: Arachidyl amido cholanoic acid; Fatty acid–bile acid conjugates

1. Introduction

Conjugation with bile acids has been used to target the hepatic uptake and biliary secretion of various drugs [1,2]. Fatty acid bile acid conjugates (FABACs) are a new class of molecules synthesized with the aim of reducing cholesterol crystallization in bile [3]. The FABACs, and particularly arachidyl amido cholic acid (aramchol, C20-FABAC), were shown to retard cholesterol crystallization [3], dissolve preformed cholesterol crystals in model bile solutions [4], as well as in native human gall bladder bile ex vivo. Aramchol administrated intragastrically prevented biliary cholesterol crystal formation in hamsters and inbred mice [4]. Moreover, it prevented gallstone formation in inbred mice and dissolved in vivo preexisting crystals or gallstones [5]. In hundreds of animals tested to date, no evidence of toxicity has been noted. Rarely, minor transaminase elevations were found, but only in animals on high fat diets [6]. Clearly aramchol has great promise to develop into a drug to prevent and/or dissolve cholesterol gallstones in humans. For this purpose formal toxicity studies have to be performed requir-

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ing accurate monitoring of aramchol levels in body fluids. The lack of a sensitive validated method to measure aramchol has precluded elucidation of the pharmacokinetics. Our goal was to develop and validate a highly sensitive method for the detection and quantification of aramchol in various matrices, i.e. plasma, bile, feces and cell culture media using liquid chromatography–tandem mass spectrometry (LC–MS–MS).

2. Materials and methods

2.1. Reagents

Chloroform and methanol were of HPLC grade, acetic acid (glacial) 100% and ammonia (25%) were purchased from Merck (Darmstadt, Germany). Purified water was obtained via a Milli-Q system (Millipore, Molsheim, France). All other reagents were of analytical grade.

2.2. Fabacs preparation

Aramchol was prepared as previously described [3]. Arachidic acid (n-$C_{20}O_2H_{40}$) or Stearic acid (n-$C_{18}O_2H_{36}$) were conjugated via an amide bond to position 3 of cholic acid. The conjugation was in the β configuration giving the final product of arachidyl amido cholic acid (aramchol, mol. wt. 700.4) or stearyl amido cholic acid (stamchol). The latter is used as the internal standard in the present method. The final products were purified by silica gel chromatography and characterized by ^{1}H nuclear magnetic resonance (NMR) and mass spectrometry. Aramchol was at least 98% pure by NMR. Stamchol showed a 2% contamination with aramchol by mass spectrometry.

2.3. Mouse experiments

Aramchol (150 mg/kg mouse/day) was suspended in saline and given by gavage to a group of six female mice from the FVB strain. A group of six control mice received 0.2 ml saline by gavage. All animals were held in standard cages at an animal facility at room temperature (22 °C), under a 12-h light/dark cycle. Water and chow were given ad libitum and animals were weighed daily. Bile was collected for 15 min by cannulation of the gall bladder under Hypnorm (fentanyl/fluanisone; 1 ml/kg) and diazepam (10 mg/kg) anesthesia, using a heat pad to maintain body temperature. At the end of the procedure, blood was collected by cardiac puncture and the animals were killed. Bile flow was determined gravimetrically. The study was approved by the animal experimentation committee of the institution.

2.4. Standard preparation

Aramchol and the internal standard (stearyl-amido-cholic acid, stamchol) were dissolved individually in chloroform at a concentration of 100 μmol/l. These solutions were kept refrigerated and were used as stock solutions.

2.5. Control standard preparation

Normal human plasma was supplemented with aramchol dissolved in methanol to final concentrations as indicated and added quickly to the plasma under continuous vortexing. Final methanol concentration was 1% (v/v) in 4 ml plasma. Samples were divided into aliquots of 100 μl and stored under −20 °C until use. These control standards were analyzed in parallel with the samples, and were used for the validation and statistical evaluation of the method.

2.6. Sample preparation

Mouse plasma and bile samples were extracted according to the Bligh and Dyer procedure [7] with slight modifications. In brief, 100 μl of plasma sample was acidified with 200 μl of 2% (v/v) acetic acid in water; 2.2 volumes of methanol and one volume of internal standard (I.S.), stamchol in chloroform was then added to the sample. The mixture was incubated under continuous mixing for 30 min at room temperature. Phase separation was achieved by adding one volume of water and one volume of chloroform followed by subsequent centrifugation for 10 min at 2000 rpm at 4 °C. The lower phase was transferred into a fresh tube and then evaporated under a stream of dry N_2 gas. The residue was

redissolved in 100 μl of chloroform. Five microliters was injected into the LC–MS–MS system.

2.7. Liquid chromatography mass spectrometry (LC–MS–MS)

LC–MS–MS was carried out with a Hewlett-Packard (Waldbronn, Germany) HP 1100 binary pump, a Gilson (Villiers-le-Bel, France) 231XL autosampler and a Micromass (Manchester, UK) Quatro II tandem mass spectrometer equipped with electrospray ionization (ESI). The LC separation was performed on Supelguard LC-Si column (5 μm, 20×4.6 mm), Supelco (Zwijndrecht, Netherlands). Eluent A was 10% (v/v) water in methanol with 0.1% (v/v) ammonia 25%. Eluent B was chloroform with 0.1% ammonia 25%. After equilibration of the column with 5% A in B, 5 μl of the sample was injected and the column was washed for 0.5 min with the starting eluent, then aramchol and internal standard were eluted with 50% A in B. After 2 min the column was re-equilibrated with 5% A in B. The flow-rate was 1.1 ml/min. MS–MS parameters were as follows: negative ion mode, capillary voltage 3.1 kV, cone voltage 70 V, collision energy 60 eV, and collision pressure 0.003 mBar. Standards and samples were measured within the stable dynamic range of the electron multiplier of the mass spectrometer. Argon was used as collision gas. Aramchol and I.S. were analyzed by multiple reaction monitoring (MRM) using the following transitions—m/z 700.4–310.3 and m/z 672.4–282.3. Calibration samples were prepared on a large scale using large volumes and glass pipettes, limiting the effect of evaporation in adjusting the exact and constant concentration

3. Results

3.1. MS–MS parameters

The MS–MS spectrum obtained by infusion of 50 μmol/l aramchol is shown in Fig. 1. Transmitting aramchol via Q_1 and scanning the second resolving

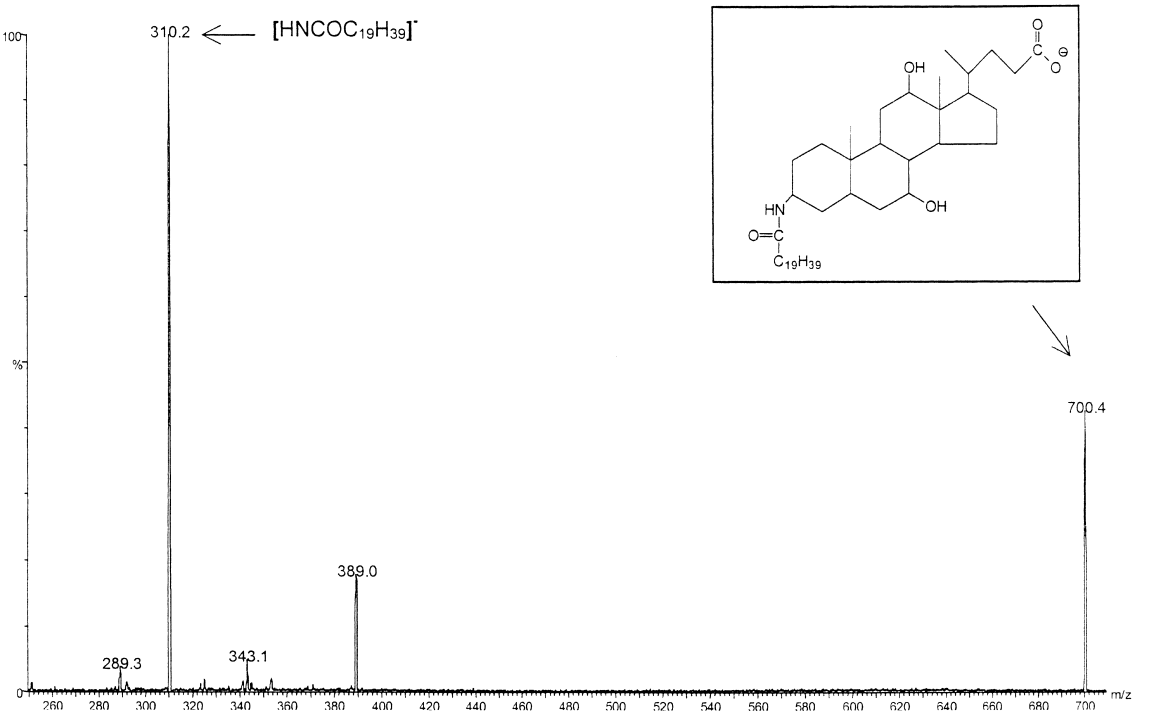

Fig. 1. MS–MS spectrum obtained by infusion of 50 μmol/l aramchol. The chromatogram shows the molecular ion at m/z 700.4, which produced the main fragment at m/z 310.2.

quadrupole (Q_3) for products generated by fragmentation in the collision cell resulted in this spectrum. With the MS–MS setting used, aramchol predominantly formed a product ion at m/z 310.3, this ion is produced by cleavage of the amide from the cholic acid skeleton to form an arachidyl-amide ion $[C_{19}H_{39}–CONH]^-$ as shown in Fig. 1. Simultaneously, the internal standard, stamchol, forms a predominant product at m/z 282.3 (stearyl-amide ion $[C_{17}H_{35}–CONH]^-$) in the same manner. The instrument parameters were optimized for maximal intensity of the selected daughter ions. These parameters were used for the acquisition file for MRM analysis. Fig. 2 shows the MRM chromatogram obtained by analysis of the mixture of aramchol (10 μmol/l) and stamchol (1.5 μmol/l). The difference in retention time between both compounds was less than 1 s making stamchol a suitable internal standard. In unspiked plasma samples no interfering peaks were observed (not shown).

3.2. Quantitative analysis

Plasma was chosen as the target for initial quantitative studies since it is the compartment of choice for monitoring drugs in general and suitable for studying the rate of aramchol absorption after oral administration.

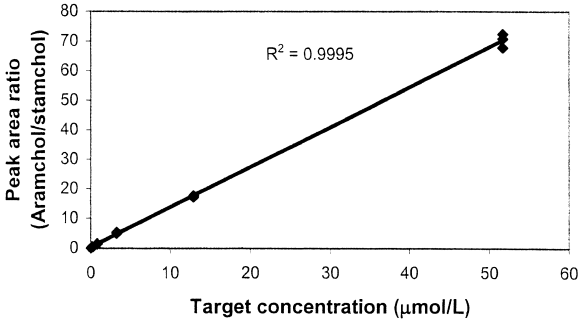

Fig. 3. Linearity of aramchol analyzed directly from plasma specimens. The equation for the line is: $y=(1.35\pm0.008)x+(0.256\pm0.186)$. Each data point represents the mean±SD ($n=4$).

The linearity of the method was evaluated for concentrations from 0 to 50 μmol/l of aramchol ($n=4$ for each concentration). As shown in Fig. 3, the method was linear in this concentration range [$y=(1.35\pm0.008)x+(0.256\pm0.186)$; $r^2=0.9995$, 95% confidence interval]. The limit of detection was 0.02 μmol/l at a signal-to-noise ratio of 5, and the limit of quantification was 0.05 μmol/l.

The measurements for within run accuracy for aramchol added to plasma are shown in Table 1.

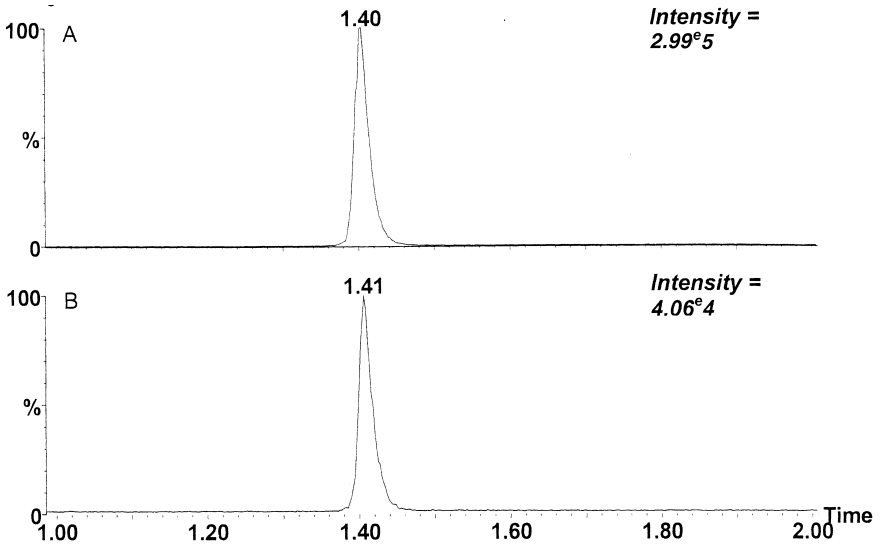

Fig. 2. Normalized MRM extracted ion chromatograms of aramchol (A, m/z 700.4→m/z 310.3) and the internal standard stamchol (B, m/z 672.4→m/z 282.3) with a calculated aramchol concentration of 10 μmol/l.

Table 1
Within run accuracy and imprecision for quantifying aramchol in control plasma samples

Target μmol/l	Measured μmol/l	SD μmol/l	C.V.%
0.1	0.09	0.008	6.4
2.3	1.92	0.05	2.6
50	43.9	2.26	5.2

$n=10$ for each concentration.

Three targeted levels of aramchol, low, intermediate and high concentrations were added to plasma specimens (final concentrations in plasma: 0.1, 2.3 and 50 μmol/l). Ten aliquots from each of the three samples were extracted as described in the Materials and methods section and aramchol was measured. The within run C.V.s were in the range 2.6–6.4%.

The precision of the method for quantifying Aramchol was evaluated over a period of several months by analyzing the same low, intermediate and high concentration controls with each batch of specimens. A typical batch consisted of plasma specimens from aramchol intragastric administered and untreated mice together with the three control plasma specimens. The concentrations of the controls were selected to demonstrate that we could reliably analyze samples in an extensive concentration range. As shown in Table 2, the imprecision was 11.4% for the lowest control, 6.2% for the intermediate control and 5.3% for the high control.

The recovery and precision data are summarized in Table 3. These experiments were conducted using as matrix human plasma. Aramchol (18 or 450 μmol/l methanolic solution) was added to 10 different human plasma samples (1 ml), corresponding to final aramchol concentrations of 0.86 or 21.1 μmol/l. Three aliquots of each set were extracted with internal standard as described above and analyzed on three consecutive days. Quantitative recovery of 93% for both levels and good precision of 5.2–5.6% were obtained, demonstrating the exactness of the present method for quantitative determinations of aramchol.

Table 2
Between run accuracy and imprecision for quantifying aramchol in control plasma samples

	Level 1	Level 2	Level 3
Mean (μmol/l)	0.10	1.94	45.0
SD (μmol/l)	0.01	0.12	2.41
C.V. (%)	11.4	6.15	5.34
Range (μmol/l)	0.09–0.12	1.76–2.09	41.4–49

Each control was analyzed on 12 different occasions, during a 3-month period.

Table 3
Recovery of aramchol from plasma samples

Added (μmol/l)	0.86	21.1
Mean±SD (μmol/l)	0.80±0.04	19.6±1.1
Recovery (%)	93	93
C.V. (%)	5.2	5.6

Aramchol, 0.86 or 21.1 μmol/ml was added to 10 different plasma samples. Each sample was analyzed on 3 consecutive days. Total $n=30$ for each concentration.

To verify whether the dynamic range of the aramchol measurements is adequate for studies in animals, we determined the compound in serum and bile of mice given aramchol in the therapeutic dose of 150 mg/kg mouse/day. The concentration in serum was 1.66±0.75, range 0.65±0.07–3.0±0.24 μmol/l. In bile the concentration was 0.875±0.19 μmol/l leading to a secretion rate of 3.5±1.0 pmol/min per 100 g body weight. In plasma and bile from control mice no aramchol was detected.

4. Discussion

In this study we developed and validated an analytical method for the quantification of aramchol levels in body fluids in general and in plasma in particular using LC–MS–MS. This method is easily adjustable for aramchol determination in other matrix samples such as bile, cell culture studies or even stools. The handling and sample preparation regardless of origin requires only minor modifications. Currently, the present method is the sole assay available for reliable determinations of aramchol in plasma.

Aramchol was shown to be a potential efficacious drug for preventing and/or dissolving of cholesterol gallstones [3–5]. As such, the monitoring of aramchol is imperative. Since the absorption of Aramchol was not expected to be very efficient and concentrations in blood were expected to be low, our calibration curve was adjusted to the micro-molar range of measurements. The use of 1.5 μmol/l

internal standard was shown to be the suitable concentration for determinations of up to 50 μmol/l aramchol. When higher concentrations are expected it is recommended to increase the levels of the internal standard. Using this method we have found a high accuracy and precision for quantifying aramchol in plasma during short and long-term experiments; both in intra and extra assays. Good recoveries for both high and low concentrations were observed.

Stamchol was used as internal standard since a stable isotope labeled aramchol, the ideal internal standard, is currently not available. Stamchol has similar physico–chemical properties as aramchol. Both compounds elute from the column with the same retention time and show similar responses in the MRM mode, which qualifies stamchol as an appropriate internal standard. Due to the strong hydrophobic properties of aramchol we were forced to use chloroform as the solvent for the chromatography although this is not the solvent of choice for electrospray ionization.

In conclusion, we present here a sensitive method to measure a novel class of bile acid conjugates, which allows determination of pharmacokinetics and biodistribution of these promising compounds.

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References

[1] W. Kramer, G. Wess, G. Schubert, M. Bickel, F. Girbig, U. Gutjahr et al., J. Biol. Chem. 267 (1992) 18598.
[2] W. Kramer, G. Wess, Eur. J. Clin. Invest. 26 (1996) 715.
[3] T. Gilat, J. Somjen G, Y. Mazur, A. LeikinFrenkel, R. Rosenberg, Z. Halpern et al., Gut 48 (2001) 75.
[4] T. Gilat, A. LeikinFrenkel, I. Goldiner, H. Laufer, Z. Halpern, F.M. Konikoff, Lipids 36 (2001) 1135.
[5] T. Gilat, A. Leikin-Frenkel, I. Goldiner, Z. Halpern, F.M. Konikoff, Hepatology 35 (2002) 597.
[6] F.M. Konikoff, A. Leikin-Frenkel, I. Goldiner, M. Michowich, E. Brezovsky, D. Harats et al., Eur. J. Gastroenterol. Hepatol. 15 (2003) 649.
[7] E.G. Bligh, W.J. Dyer, Can. J. Biochem. Physiol. 37 (1959) 911.