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An ultra-high performance liquid chromatography–tandem mass spectrometric assay for quantifying 3-ketocholanoic acid: Application to the human liver microsomal CYP3A-dependent lithocholic acid 3-oxidation assay

Sumit Bansal[a], Swee Fen Chai[a], Aik Jiang Lau[a,b,*]

[a] Department of Pharmacy, Faculty of Science, National University of Singapore, Singapore
[b] Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

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ABSTRACT

Lithocholic acid (LCA), a hepatotoxic and carcinogenic bile acid, is metabolized to 3-ketocholanoic acid (3-KCA) by cytochrome P450 3A (CYP3A). In the present study, the objectives were to develop and validate an ultra-high performance liquid chromatography-tandem mass spectrometric (UPLC–MS/MS) method to quantify 3-KCA and apply it to the human liver microsomal CYP3A-dependent LCA 3-oxidation assay. Chromatographic separation was achieved on a Waters ACQUITY^{TM} UPLC C_{18} column (50 × 2.1 mm, 1.7 μm) with a gradient system consisting of 0.1% v/v formic acid in water (solvent A) and 0.1% v/v formic acid in acetonitrile (solvent B). The retention time was 3.73 min for 3-KCA and 2.73 min for cortisol (internal standard). Positive electrospray ionization with multiple reaction monitoring (MRM) mode was used to quantify 3-KCA (m/z 375.4 → 135.2) and cortisol (m/z 363.5 → 121.0). The limit of detection of 3-KCA was 10 μM, the lower limit of quantification was 33.3 μM, and the calibration curve was linear from 0.05–10 μM with $r^2 > 0.99$. Intra-day and inter-day accuracy and precision were <13.7%. The quality control samples were stable when assessed after 4 h at room temperature, 24 h at 4 °C, 14 days at −20 °C, and three freeze-thaw cycles. The liver microsomal matrix did not affect 3-KCA quantification. The amount of KCA formed in the human liver microsomal LCA 3-oxidation assay was linear with respect to the amount of microsomal protein (up to 40 μg) and incubation time (5–30 min). Enzyme kinetics experiment indicated that LCA 3-oxidation followed the Michaelis-Menten model with an apparent K_m of 26 ± 7 μM and V_{max} of 303 ± 50 pmol/min/mg protein. This novel UPLC–MS/MS method for quantifying 3-KCA offers a specific, sensitive, and fast approach to determine liver microsomal LCA 3-oxidation.

1. Introduction

Bile acids are steroidal chemicals that play important physiological roles in the intestinal absorption of dietary fats, fat-soluble vitamins, and lipid-soluble drugs, and elimination of cholesterol [1]. Primary bile acids, such as cholic acid and chenodeoxycholic acid, are synthesized in the liver and secreted or transported into bile and intestines. These primary bile acids are metabolized to secondary bile acids, such as deoxycholic acid and lithocholic acid (LCA), by 7α-dehydroxylase present in intestinal bacteria. Among the various bile acids, LCA, which is a metabolite of chenodeoxycholic acid, represents approximately 4.5% of the total bile acids in human liver [2]. Despite its relatively low composition in bile, LCA is the most toxic bile acid [3,4]. Increased levels of LCA have been reported to result in cholestasis in rats [5], hepatotoxicity in mice, rats, and hamsters [3,6–8], and promotes colon carcinogenesis in a murine carcinogenesis model [9].

LCA-mediated toxicity can be attenuated by increasing LCA hepatic biotransformation and elimination. The biotransformation of LCA involves oxidation by cytochrome P450 (CYP) enzymes, such as CYP3A [10–13], and conjugation by phase II drug-metabolizing

Abbreviations: CYP, cytochrome P450; CYP3A, cytochrome P450 3A; DMSO, dimethyl sulfoxide; HPLC–UV, high-performance liquid chromatography–ultraviolet spectroscopy; 3-KCA, 3-ketocholanoic acid; LC–MS/MS, liquid chromatography–tandem mass spectrometry; LCA, lithocholic acid; $MgCl_2$, magnesium chloride; MRM, multiple reaction monitoring; NADPH, reduced β-nicotinamide adenine dinucleotide 2′-phosphate; UPLC–MS/MS, ultra-high performance liquid chromatography–tandem mass spectrometry.

* Corresponding author at: Department of Pharmacy, Faculty of Science, National University of Singapore, 18 Science Drive 4, 117543, Singapore.
E-mail address: aikjiang.lau@nus.edu.sg (A.J. Lau).

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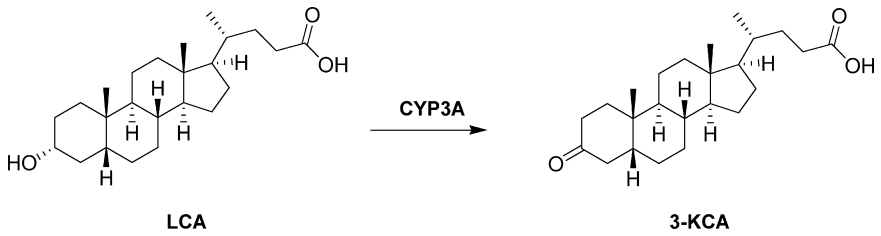

Fig. 1. Chemical structures of LCA and 3-KCA.

enzymes, such as sulfotransferase 2A1 [14,15] and uridine 5′-diphospho-glucuronosyltransferase 1A3 [16]. In human liver microsomes, LCA is metabolized predominantly by cytochrome P450 3A (CYP3A) to hyodeoxycholic acid, murideoxycholic acid, and chenodeoxycholic acid (in descending order of abundance) [10,11]. In another two studies, 3-ketocholanoic acid (3-KCA), which is also known as 3-oxo-5β-cholan-24-oic acid, dehydrolithocholic acid, or 3-ketolithocholic acid, is the major oxidative metabolite formed by human recombinant CYP3A4 [12] and human liver microsomes [13]. According to detailed reaction phenotyping, CYP3A4, but not CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A5, or CYP4A11, catalyzes the biotransformation of LCA to 3-KCA (Fig. 1) [13]. Interestingly, 3-KCA was reported to activate pregnane X receptor [17], vitamin D receptor [18], and farnesoid X receptor [18] to a greater extent than LCA, thereby inducing CYP3A and other drug-metabolizing enzymes and increasing the metabolism of LCA or other bile acids. Due to the role of 3-KCA as a regulator of bile acid metabolism and homeostasis, it is important to have an accurate and precise method in the quantification of 3-KCA and liver microsomal CYP3A-dependent LCA 3-oxidation.

To date, analytical methods for the quantification of 3-KCA are limited. 3-KCA in incubation samples containing recombinant CYP3A4 enzymes was first identified and detected using GC–MS [12], which required sample derivatization and tedious sample preparation/extraction method. Subsequently, a HPLC–MS method with selected ion monitoring was developed for quantification of 3-KCA in rat/human liver microsomal samples [13,19]. However, this single quadrupole MS method is not as specific or sensitive as compared to a tandem MS method with multiple reaction monitoring (MRM). In addition, the HPLC system required a longer run time as compared to an ultra-high performance liquid chromatography (UPLC) system. Therefore, in the present study, the aims were to: (1) develop and validate an UPLC–MS/MS method for the quantification of 3-KCA; (2) apply the analytical method to characterize the linearity of the human hepatic microsomal CYP3A-dependent LCA 3-oxidation assay with respect to the amount of liver microsomes and incubation time; (3) determine the enzyme kinetics of human hepatic microsomal LCA 3-oxidation. Our UPLC–MS/MS method offers a specific, sensitive, and fast approach for quantifying 3-KCA and its application to the liver microsomal CYP3A-dependent LCA 3-oxidation.

2. Materials and methods

2.1. Chemicals, reagents, and enzymes

LCA (CAS #434-13-9) and 3-KCA (CAS #1553-56-6) were purchased from Steraloids, Inc. (Newport, RI, USA). Cortisol (also known as hydrocortisone; CAS #50-23-7), β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH), magnesium chloride hexahydrate ($MgCl_2$), and dimethyl sulfoxide (DMSO) were bought from Sigma-Aldrich (St. Louis, MO, USA). Di-potassium hydrogen phosphate and formic acid (98–100%) were purchased from VWR International (Singapore), and potassium dihydrogen phosphate was from Merck Millipore Division, Merck Pte. Ltd. (Singapore). Methanol (HPLC grade) and acetonitrile (HPLC grade) were bought from Tedia Company, Inc. (Fairfield, OH, USA). Ultra-pure water was obtained using Direct-Q® 3 UV Water Purification System (Merck Millipore). Human liver microsomes (catalog #452156; lot #88114), which were pooled from 50 individual donors, were purchased from Corning Gentest (Corning, NY, USA).

2.2. Instrumentation and UPLC–MS/MS conditions

The UPLC–MS/MS system consisted of an ACQUITY™ UPLC system (Waters, Milford, MA, USA) coupled to an AB Sciex 3200 QTRAP® mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a TurboIonSpray® ion source. Chromatographic separation was achieved using a Waters ACQUITY™ UPLC BEH C_{18} column (50 × 2.1 mm i.d., 1.7 μm). The column and the autosampler compartment were maintained at 30 °C and 4 °C, respectively. The flow rate was 0.5 ml/min and the sample injection volume was 5 μl. The mobile phases were (A) water containing 0.1% v/v formic acid and (B) acetonitrile containing 0.1% v/v formic acid. The gradient conditions were as follows: 20% B from 0.0–1.0 min, linear increase to 95% B from 1.0–2.5 min, 95% B from 2.5–5.0 min, linear decrease to 20% B from 5.0–5.1 min, and 20% B from 5.1–6.0 min. The total run time was 6 min. The UPLC effluent was diverted to the waste before 1.1 min and after 5.0 min.

The mass spectrometer was operated in the positive electrospray ionization mode. Nitrogen gas was used as the curtain gas, collision gas, and ion source gas. The ion source parameters were: spray voltage, 5500 V; ion source temperature, 550 °C; curtain gas, 10 psi; collision gas setting, medium; ion source gas 1, 50 psi; and ion source gas 2, 50 psi. The optimized compound-dependent MS parameters for 3-KCA were: 46 V declustering potential, 4 V entrance potential, 14 V collision cell entrance potential, 45 V collision energy, 2.5 V collision cell exit potential, and 100 ms dwell time. The optimized compound-dependent MS parameters for cortisol were: 50 V declustering potential, 4.62 V entrance potential, 17 V collision cell entrance potential, 36 V collision energy, 3 V collision cell exit potential, and 100 ms dwell time. 3-KCA and cortisol (internal standard) were determined in the MRM mode. The MRM transition for 3-KCA was m/z 375.4 → 135.2 and that for cortisol was m/z 363.5 → 121.0, respectively. Data acquisition and processing were performed using Analyst software version 1.4.2 (Applied Biosystems).

2.3. Calibration standards and quality control samples

A primary stock solution of authentic 3-KCA was prepared at a concentration of 100 mM in DMSO and stored at −20 °C. Each 3-KCA standard sample was freshly prepared by adding 0.2 μl of 3-KCA working standard solution (0.05–10 mM; diluted in DMSO) to a 200 μl incubation mixture containing potassium phosphate buffer (50 mM; pH 7.4), $MgCl_2$ (3 mM), LCA (30 μM), and heat-inactivated