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Indocyanine green clearance varies as a function of N-acetylcysteine treatment in a murine model of acetaminophen toxicity

Alessandra Milesi-Hallé[a,c,d], Susan M. Abdel-Rahman[e,f], Aliza Brown[a,c,d], Sandra S. McCullough[a,c,d], Lynda Letzig[a,c,d], Jack A. Hinson[b,c], Laura P. James[a,b,c,d,*]

[a] Department of Pediatrics, Little Rock, AR, United States
[b] Department of Pharmacology and Toxicology, Little Rock, AR, United States
[c] University of Arkansas for Medical Sciences, Little Rock, AR, United States
[d] Arkansas Children's Hospital Research Institute, Little Rock, AR, United States
[e] Children's Mercy Hospital and Department of Pediatrics, Kansas City, MO, United States
[f] University of Missouri-Kansas City School of Medicine, Kansas City, MO, United States

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ABSTRACT

Standard assays to assess acetaminophen (APAP) toxicity in animal models include determination of ALT (alanine aminotransferase) levels and examination of histopathology of liver sections. However, these assays do not reflect the functional capacity of the injured liver. To examine a functional marker of liver injury, the pharmacokinetics of indocyanine green (ICG) were examined in mice treated with APAP, saline, or APAP followed by N-acetylcysteine (NAC) treatment.Male B6C3F1 mice were administered APAP (200 mg/kg IP) or saline. Two additional groups of mice received APAP followed by NAC at 1 or 4 h after APAP. At 24 h, mice were injected with ICG (10 mg/kg IV) and serial blood samples (0, 2, 10, 30, 50 and 75 min) were obtained for determination of serum ICG concentrations and ALT. Mouse livers were removed for measurement of APAP protein adducts and examination of histopathology. Toxicity (ALT values and histology) was significantly increased above saline treated mice in the APAP and APAP/NAC 4 h mice. Mice treated with APAP/NAC 1 h had complete protection from toxicity. APAP protein adducts were increased in all APAP treated groups and were highest in the APAP/NAC 1 h group. Pharmacokinetic analysis of ICG demonstrated that the total body clearance (Cl_T) of ICG was significantly decreased and the mean residence time (MRT) was significantly increased in the APAP mice compared to the saline mice. Mice treated with NAC at 1 h had Cl_T and MRT values similar to those of saline treated mice. Conversely, mice that received NAC at 4 h had a similar ICG pharmacokinetic profile to that of the APAP only mice. Prompt treatment with NAC prevented loss of functional activity while late treatment with NAC offered no improvement in ICG clearance at 24 h. ICG clearance in mice with APAP toxicity can be utilized in future studies testing the effects of novel treatments for APAP toxicity.

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1. Introduction

Acetaminophen (paracetamol, N-acetyl-*p*-aminophenol, APAP) is the most commonly used antipyretic and analgesic agent in the world today. Despite its safety at therapeutic doses, APAP is a major cause of acute liver failure in the United States and worldwide [1,2].

The toxicity of APAP is characterized by the pathologic appearance of centrilobular necrosis. Determination of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in serum and histopathologic examination of liver tissues are the common measures used to assess APAP toxicity in the experimental setting. Elevated hepatic transaminase levels are used as a measure of hepatocyte lysis and thus do not reflect the functional capacity of the liver. Because structurally injured hepatocytes may be partially functional [3,4], it is important to distinguish liver cell injury from loss of functional capacity. Assays that characterize liver function are critical in studies that assess hepatic recovery following toxin-mediated and other causes of liver injury. For example, a useful endpoint in the design of potential new therapies for liver toxicity would be demonstration of return to baseline liver function.

Abbreviations: ALT, alanine aminotransferase; APAP, acetaminophen, paracetamol, N-acetyl-*p*-aminophenol; AST, aspartate aminotransferase; AUC, area under the curve; $AUC_{0-\infty}$, AUC to infinity; Cl_T, total body clearance; C_n, final plasma concentration; λz, apparent terminal elimination rate constant; ICG, indocyanine green; GSH, glutathione; MRT, mean residence time; NAC, N-acetylcysteine; NAPQI, N-acetyl-*p*-benzo-quinone imine.

* Corresponding author at: Section of Clinical Pharmacology and Toxicology, Department of Pediatrics, 3 Children's Way, Slot 512-23, Arkansas Children's Hospital, Little Rock, AR 72202, United States. Tel.: +1 501 364 1418; fax: +1 501 364 3654.
E-mail address: jameslaurap@uams.edu (L.P. James).

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Indocyanine green (ICG), a water-soluble dye that does not undergo metabolism or enterohepatic circulation, serves as a measure of hepatic function because of its exclusive elimination via biliary excretion [5]. In addition, ICG distributes into the plasma volume without extravascular distribution [6]. The clearance of ICG has been used as a measurement of hepatic blood flow and liver function in clinical studies [7–9]. In addition, the clearance of ICG is used to evaluate hepatic function pre- and post-liver transplantation [10] and recent studies have found ICG to be a more reliable indicator of liver function post-operatively than other conventional endogenous markers such as bilirubin and prothrombin time [11]. Several studies performed in experimental models have examined the clearance of ICG as a functional marker of liver injury [6,12,13]. However, the clearance of ICG has not been previously studied as an endpoint to assess the effectiveness of treatments for drug-induced toxicity.

N-acetylcysteine (NAC) is a thiol-containing compound that has been used for over 30 years as the antidote for APAP toxicity in man [14]. NAC acts as a glutathione (GSH) precursor, promoting GSH synthesis and increasing hepatic GSH stores in order to detoxify N-acetyl-*p*-benzoquinone imine (NAPQI), the reactive intermediate metabolite of APAP [15,16]. In addition, NAC may react directly with NAPQI and acts as a scavenger of the reactive oxygen/nitrogen species peroxynitrite. Treatment with NAC has been shown to reduce APAP-induced liver toxicity in humans if administered within 10 h of an APAP overdose [17]. NAC has an intermediate hepatoprotective effect if administered after 10 but prior to 24 h of APAP overdose [17]. Additional studies have shown that NAC treatment improves patient outcome even when administered late (e.g., more than 24 h post-overdose [18,19]), despite having minimal effects on the hepatic injury, *per se*.

We hypothesized that ICG could be used as a functional marker of APAP toxicity in the mouse and may represent a potential assay to assess the efficacy of various novel interventions for the treatment of APAP toxicity. To this end, we characterized the pharmacokinetics of ICG in APAP-treated mice and compared these parameters to those of saline-treated mice. In addition, since NAC is the current cornerstone of management of APAP toxicity, the pharmacokinetics of ICG were examined in mice following NAC treatment. Two treatment schedules for NAC were tested to mimic the clinical setting of prompt and delayed treatment with NAC [17]. The data presented herein demonstrate that the pharmacokinetics of ICG differ among saline treated mice, APAP treated mice, and APAP treated mice that receive early or late NAC treatment.

2. Materials and methods

2.1. Drugs and reagents

APAP, ICG (Cardiogreen) and NAC were purchased from Sigma Chemical Co. (St. Louis, MO). All chemicals were of reagent grade or better.

2.2. Animals

B6C3F1 mice (six-week-old males; average weight of 25.1 g) were purchased from Harlan Sprague–Dawley (Indianapolis, IN). One week before the initiation of experiments, mice were acclimatized to the facility and placed on a 12 h light/dark cycle under controlled ambient temperature. Animals were housed 3 per cage (APAP/ICG studies) or in individual cages (APAP/NAC/ICG studies); they were fed *ad libitum* and fasted overnight prior to all studies. All animal experimentation was in agreement with the criteria of the "Guide for the Care and Use of Laboratory Animals" as per the guidelines of the National Academy of Sciences. Experimental protocols were reviewed and approved by University of Arkansas for Medical Sciences Animal Care and Use Committee.

2.3. Experimental protocol

Animals were segregated into four treatment groups as follows: (1) saline (0.9% NaCl; $n = 6$), (2) APAP (200 mg/kg in saline; $n = 8$), (3) APAP (200 mg/kg in saline) followed by NAC (1200 mg/kg IP in saline, pH 7.4) at 1 h ($n = 6$), and (4) APAP (200 mg/kg in saline) followed by NAC at 4 h ($n = 7$) given via IP injection. At 24 h after APAP or saline, mice received ICG (10 mg/kg) as a 0.2 ml bolus via tail vein injection. Mice were then anesthetized in a CO_2 chamber before blood was drawn from the retro-orbital vein. Blood samples for determination of ICG concentrations in serum were obtained at baseline and at 2, 10 and 30 min (control group) or at 2, 10, 30, 50 and 75 min (APAP groups) after ICG administration. The total amount of blood sampled from each mouse was less than 15% of the total circulating blood volume [20] to avoid hemodynamic changes that could potentially affect the pharmacokinetics of ICG. Blood samples were allowed to coagulate at room temperature and then centrifuged to obtain serum. Euthanasia was conducted by cervical dislocation and livers were quickly removed for histopathological analyses. All samples were stored at −80 °C until analysis.

2.4. Toxicity assays and liver histopathology

Serum ALT levels were determined using an Ace Alera Chemistry Analyzer (Alfa Wassermann Inc., West Caldwell, NJ). Hematoxylin and eosin staining was performed for histological examination of mouse livers. The extent of necrosis in liver sections was quantified by outlining the necrotic areas with the interactive spline measuring tool in the AxioVision 4.6.3 program (Carl Zeiss Inc., Germany). Three images were obtained from each section at 10× magnification. Quantification of the extent of necrosis was expressed as a percentage of the entire histological field [21].

2.5. Measurement of ICG in serum by high performance liquid chromatography

Analysis of ICG concentrations in mouse serum was performed using a Waters Alliance 2695 Separations Module with a photodiode array detector set at 720 nm. The method was adapted from Kulkarni and Pegram [12] and validated in our laboratory. A Waters C_{18} symmetry column equipped with a guard column (Waters Associates, Milford, MA) was used for reverse-phase chromatography at a temperature of 35 °C. The mobile phase consisted of 50 mM potassium phosphate buffer (pH 5.52): acetonitrile (55:45), at a flow rate of 1.0 ml/min and 17-min run time. A volume of 20 μl was injected following precipitation with acetonitrile. The retention time of ICG was approximately 12 min.

2.6. Pharmacokinetic analysis

ICG serum concentration vs. time data were evaluated using a model independent approach. The pharmacokinetic profiles were curve fit using a peeling algorithm to generate initial polyexponential parameter estimates. Final estimates of the terminal elimination rate constant (λz) were determined from an iterative, nonlinear least squares regression algorithm. The area under the serum concentration versus time curve (AUC) was determined using the log-linear trapezoidal rule. Extrapolation of the AUC to infinity ($AUC_{0-\infty}$) was calculated by summation of $AUC_{0-n} + C_n/\lambda z$, where λz is the apparent terminal elimination rate constant and C_n represents the final plasma concentration predicted from the fitted apparent terminal elimination phase. Mean residence time was calculated by dividing the area under the moment curve (AUMC) by the area under the curve [MRT = AUMC/AUC].

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