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Immunoassays for Trace Chemical Analysis

Monitoring Toxic Chemicals in Humans, Food, and the Environment

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Chapter 29

Sensitive Immunochemical Assays for Monitoring Acetaminophen Toxicity in Humans

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Acetaminophen (paracetamol) is a commonly used analgesic which is hepatotoxic at high doses in humans and in laboratory animals. Toxicity is believed to be mediated by the reactive metabolite N-acetyl-p-benzoquinone imine which binds to protein thiols as 3-(cystein-S-yl)acetaminophen adducts. Ultrasensitive immunoassays for 3-(cystein-S-yl)acetaminophen derivatives were developed and extensively characterized. Using these assays the formation of this adduct in protein has been correlated with the development of the hepatotoxicity in mice and humans. In mice, adduct levels in the liver reached maximal levels at 2-4 hours and then exhibited a marked decrease which was inversely correlated with parallel elevations in serum adducts and serum levels of the liver-specific transaminase ALT. This suggested that the serum adducts were of hepatic origin and could be monitored as a biomarker of acetaminophen toxicity. Analysis of serum samples from acetaminophen overdose patients demonstrated a positive correlation between immunochemically detectable serum adducts and hepatotoxicity.

Acetaminophen (APAP, N-acetyl-p-aminophenol, paracetamol) is a widely used over-the-counter analgesic. At therapeutic doses it is a safe drug. However, at high doses it may produce severe hepatic necrosis and has also been reported in some individuals to be nephrotoxic (1-3). Available evidence indicates that acetaminophen hepatotoxicity is not a result of the parent compound but is mediated by a reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI). This metabolite is the two-electron oxidation product of acetaminophen and is formed by the microsomal cytochrome P-450 mixed function oxidase system (4-8). Following a therapeutic dose of acetaminophen the reactive metabolite is detoxified

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by reaction with the cysteine-containing tripeptide glutathione (GSH) to form 3-(glutathion-S-yl)acetaminophen. Following an acetaminophen overdose, hepatic GSH levels are depleted and covalent binding of reactive metabolite to cellular proteins correlates with the development of the hepatotoxicity (9,10). Covalent binding is believed to be primarily a reaction of NAPQI with cysteinyl sulfhydryl groups to produce the corresponding 3-(cystein-S-yl)acetaminophen (3-Cys-A)-protein adduct (11,12).

We perceived the need for sensitive assays that do not rely on the use of radioisotopes or extensive analytical methodology and that could accurately detect protein-bound acetaminophen in biological fluids in the presence of unbound acetaminophen. To this end, we recently developed sensitive avidin biotin-amplified ELISA (A-B ELISA) and particle concentration fluorescence immunoassays (PCFIA) which use antiserum specific for the major acetaminophen-protein adduct associated with toxicity (13-16). These assays are new tools to study the relation between formation of the 3-Cys-A protein adduct and acetaminophen-induced toxicity. In this report we review how these assays were developed, validated in laboratory animals, and used to quantify 3-Cys-A protein adduct formation in human acetaminophen overdose patients.

Development of Immunoassays for Protein-bound Acetaminophen

Previous assays for acetaminophen covalently bound to protein required the use of radiolabeled acetaminophen which was detected after extensive solvent extraction of the protein to remove unbound radioactivity and subsequent quantification of radiolabel associated with the protein (10). Since it was shown that acetaminophen-binding to protein is primarily via cysteine residues (3-Cys-A) (11,12), an immunogen was synthesized which contained the acetaminophen-cysteine adduct. Synthetic NAPQI was allowed to react with N-acetylcysteine to produce 3-(N-acetylcystein-S-yl)acetaminophen (NAC-acetaminophen) which was purified by HPLC. The conjugate was subsequently coupled to an immunogenic carrier protein, keyhole limpet hemocyanin (KLH), using 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride as a coupling reagent. Rabbits were immunized with the resulting KLH-NAC-acetaminophen (13).

An antigen was also synthesized for use as a solid-phase coating antigen in the A-B ELISA and as particle-bound antigen in the PCFIA. The requirement was for an immobilized antigen that contained acetaminophen bound to cysteinyl groups in protein as occurs in acetaminophen toxicity. Metallothionein was selected because it contained a high molar content of free cysteine sulfhydryl groups and it was thought that it would react directly with synthetic NAPQI to yield the relevant 3-Cys-A adduct. The synthetic scheme used to prepare the immunogen and solid phase antigen is presented in Figure 1 (13).

To select the antiserum best suited for detection of 3-Cys-A adducts in the presence of free acetaminophen, the relative inhibitory potencies of

NAC-acetaminophen and acetaminophen were compared for nine antisera in competitive A-B ELISA. For all responding rabbits, NAC-acetaminophen was a more efficient inhibitor than free acetaminophen. The relative efficiency of NAC-acetaminophen and acetaminophen to inhibit binding of antibody to solid phase metallothionein-acetaminophen are presented in Figure 2. Inhibition curves using polyclonal rabbit anti 3-Cys-A from one of the rabbits at a dilution of 1:4308, and NAC-acetaminophen and acetaminophen as inhibitors, demonstrated that NAC-acetaminophen was detected with 3.8 orders of magnitude greater sensitivity than the free drug.

Immunochemical Quantification of Acetaminophen Adducts

In initial work to characterize the epitope and to assay samples from acetaminophen-dosed animals, the competitive A-B ELISA was utilized (14). In subsequent work the assay was modified to adapt it to a competitive PCFIA format (15). The PCFIA has advantages over ELISA including: a covalently coupled solid phase, shorter incubation times, the availability of internal standards, and a more flexible assay format. It utilizes a fluorimeter and specially designed assay plates (Baxter Healthcare Corp., Mundelein, IL). Solid phase antigen for PCFIA was prepared by coupling metallothioin-acetaminophen to amino-substituted polystyrene beads using N-succinimidyl 3-(2-pyridyldithio)propionate as a coupling reagent (Figure 3). In both assays, a limiting amount of rabbit anti 3-Cys-A antibody was incubated with either 3-(N-acetyl-L-cystein-S-yl)acetaminophen standard or an unknown sample (mouse liver fraction, serum, or structurally related inhibitor) and then with solid phase acetaminophen-derivatized metallothionein. Detection of rabbit anti 3-Cys-A antibody bound to solid phase metallothioin-acetaminophen assay antigen was accomplished using avidin-biotin-horseradish peroxidase amplification and substrate conversion in the A-B ELISA, and using fluorescein isothiocyanate conjugated second antibody in the PCFIA.

To evaluate the amount of acetaminophen bound to proteins, utilizing the competitive A-B ELISA or PCFIA, inhibition by unknown samples was compared with an assay standard prepared by derivatizing protein with NAPQI. For some experiments, 3-(N-acetyl-L-cystein-S-yl)acetaminophen was used as an assay standard, in which case, the values obtained were corrected for differences in the relative inhibitory potency of 3-(N-acetyl-L-cystein-S-yl)acetaminophen and 3-Cys-A protein adduct (120 fmol/well and 2300 fmol/well, respectively) (14). After dialysis, unknown samples were diluted to a final concentration of approximately 4 μg protein/assay well, assayed in duplicate, and expressed as nmoles of 3-Cys-A per mg of protein. The ELISA and PCFIA were shown to have similar limits of detection (20 pmole/mg protein) and to recognize the same epitope as demonstrated by similar relative inhibitory potencies for N-acetylcysteine-acetaminophen, acetaminophen-bound

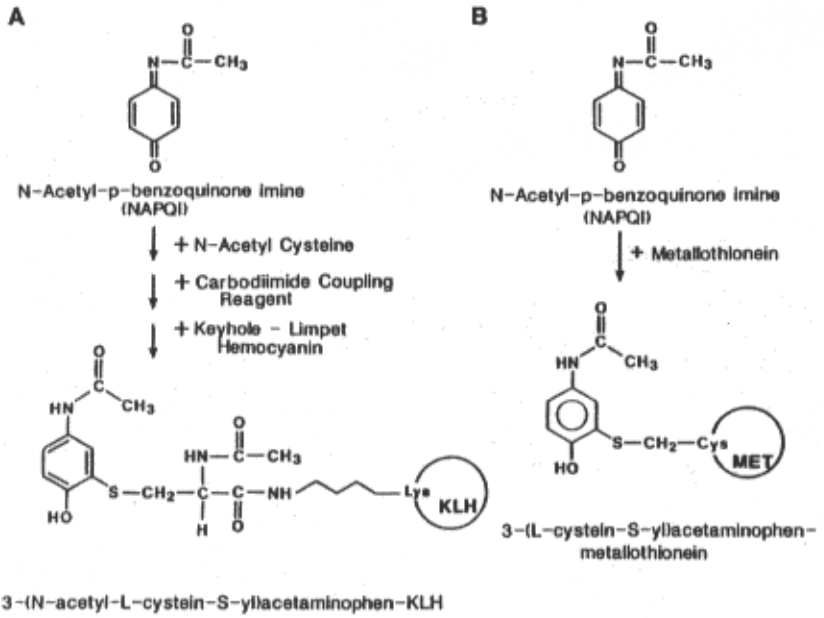


Figure 1. A. Preparation of 3-Cys-A-KLH immunogen. B. Preparation of metallothionein-acetaminophen assay antigen. Detailed methods for the syntheses are described in 13 (Reproduced from Ref. 13).

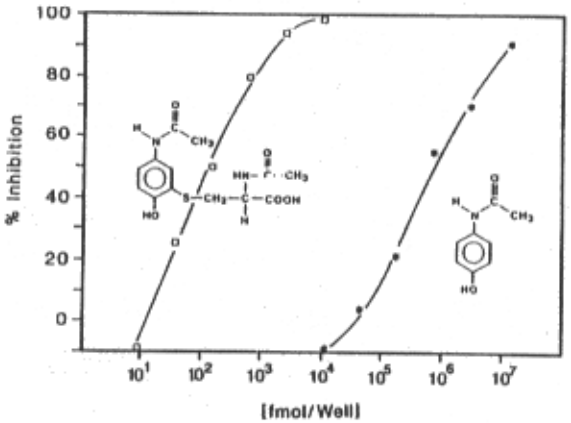
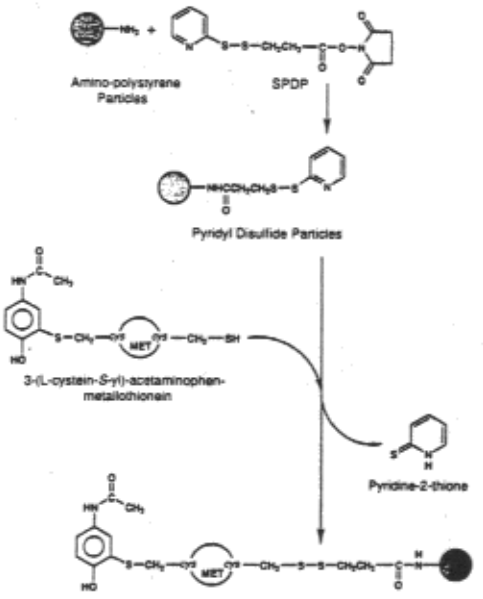


Figure 2. Relative efficiency of NAC-acetaminophen (◻) and acetaminophen (*) in the competitive A-B ELISA. The relative efficiency of NAC-acetaminophen and acetaminophen to compete for a limited amount of rabbit anti KLH-NAC-acetaminophen antibodies were determined in the presence of excess metallothionein-acetaminophen adsorbed in wells of 96-well polystyrene assay plates. The 50% inhibitory concentration was 110 fmole/well for NAC-acetaminophen and 687,000 fmole/well for acetaminophen (Reproduced from Ref. 13).



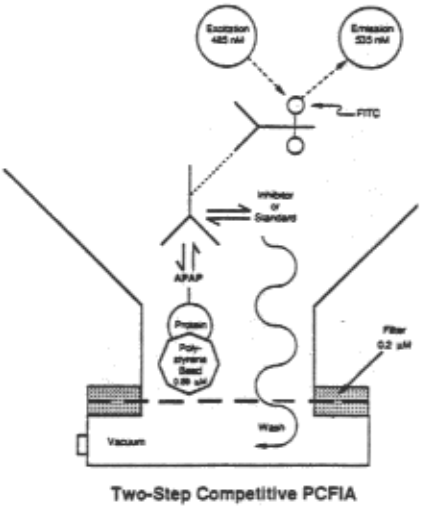


Figure 3. Assay development. Preparation of the particle-bound solid phase assay antigen, 3-Cys-A-metallothionein covalently bound to 0.89 μm amino polystyrene particles (Met-APAP modified polystyrene assay particles) and development of a PCFIA for the 3-Cys-A protein adduct (Reproduced from Ref. 15).

glutathione S-transferase, and acetaminophen in both assays. Serum samples and liver fractions containing 3-Cys-A protein adducts assayed by ELISA and PCFIA produced similar results (r= 0.89) (15).

Epitope Characterization

One of the primary reasons for development of an immunoassay that recognized acetaminophen bound to protein was to examine acetaminophen-toxicity in human overdose patients. It was therefore essential to fully characterize the epitope recognized in the assay and to evaluate the degree to which metabolites, structural analogs or other analgesics might cross react. To determine the nature of the epitope and to quantify the relative importance of specific substituent groups, twenty structurally related compounds were evaluated as inhibitors in the competitive immunoassay. These data are presented in Table I. and Figure 4 (14).

The most effective inhibitor was 3-(N-acetyl-L-cystein-S-yl)acetaminophen which had an observed 50% inhibitory concentration of 120 $\pm$ S.D. 30 fmol/well (n=19). Approximately 6,200-fold higher concentrations of unbound acetaminophen and 5.2 x 10^6-fold higher concentrations of N-acetyl-L-cysteine were required for comparable inhibition. It was demonstrated with acetaminophen analogs, that the hydroxyl group and the N-acetyl moiety of acetaminophen were important in epitope recognition. A 5,000-fold decrease in detection was observed when the analog did not contain the hydroxyl group or when the N-acetyl moiety was replaced with a hydroxyl substituent. Recognition by antibody was also dependent upon the stereochemistry of the analogs. The 50% inhibitory concentration for 3-(L-cystein-S-yl)acetaminophen was 2,300 fmol/well, whereas a 25-fold higher concentration of 3-(D-cystein-S-yl)acetaminophen was required for 50% inhibition. Although 3-(glutathion-S-yl)acetaminophen was an efficient inhibitor at very low concentrations, the 50% inhibitory concentration for GSH was 2.3 x 10^9 and for S-methylglutathione was 2.6 x 10^9 fmol/well. Other metabolites of acetaminophen were poor inhibitors in the immunoassay. 3-Hydroxyacetaminophen was nearly 2-fold less efficient than acetaminophen and over 10,000-fold less efficient than 3-(N-acetyl-L-cystein-S-yl)acetaminophen. The acetaminophen sulfate and acetaminophen glucuronide were ineffective competitive inhibitors at concentrations below 10^6 fmol/well. Other analgesics such as aspirin and phenacetin were not inhibitory even at high concentrations (Table 1) (14). Collectively, these data indicate that primary antibody specificity involves antigenic determinants found on acetaminophen bound covalently via carbon 3 to the sulfur of cysteine residues, that are not found on protein alone or on free acetaminophen (Figure 4).

This antiserum has also been used to detect acetaminophen-protein adducts in Western blots of serum and liver fractions from acetaminophen-dosed animals (16) and to localize the 3-Cys-A adduct in target tissues (17 and 18, Bucci et al. this volume).

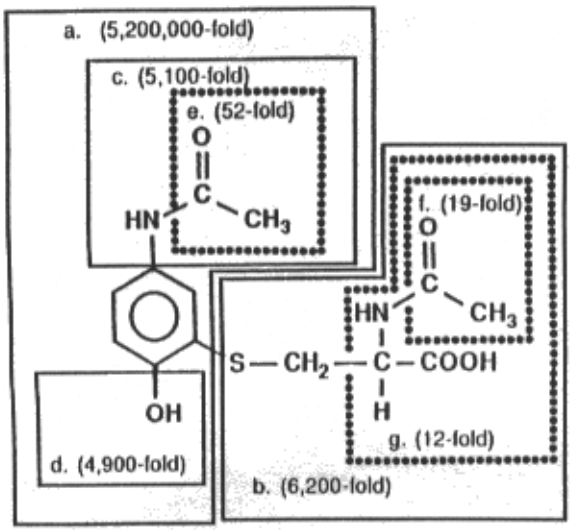


Figure 4. Effect of acetaminophen-conjugate substituents on antibody recognition in the acetaminophen-adduct immunoassay. The decrease in inhibition due to particular substituents is shown. These values were calculated by comparing the ability of the following acetaminophen-conjugate analogs to competitively inhibit antibody binding to solid-phase acetaminophen-bound metallothionein. a: N-acetyl-L-cysteine compared to 3-(N-acetyl-L-cystein-S-yl)acetaminophen. b: acetaminophen compared to 3-(N-acetyl-L-cystein-S-yl)acetaminophen. c: 2-(N-acetyl-L-cystein-S-yl)hydroquinone compared to 3-(N-acetyl-L-cystein-S-yl)-acetaminophen. d: 3-(methylthio)acetanilide compared to 3-(methylthio)acetaminophen. e: 2-(L-cystein-S-yl)-p-aminophenol compared to 3-(L-cystein-S-yl)acetaminophen. f: 3-(L-cystein-S-yl)acetaminophen compared to 3-(N-acetyl-L-cystein-S-yl)acetaminophen. g: 3-(methylthio)acetaminophen compared to 3-(N-acetyl-L-cystein-S-yl)-acetaminophen (Reproduced from Ref. 14).

Table I. Competitive Inhibition of Acetaminophen Analogs in the Acetaminophen-Adduct Assay

Inhibitor	50% Inhibition (fmol/well)
3-(N-acetyl-L-cystein-S-yl)acetaminophen	120
3-(glutathion-S-yl)acetaminophen	300
3-(methylthio)acetaminophen	1,400
3-(L-cystein-S-yl)acetaminophen	2,300
3-(diglutathion-S-yl)diacetaminophen	22,000
3-(D-cystein-S-yl)acetaminophen	57,000
3-(glutathion-S-yl)diacetaminophen	75,000
2-(L-cystein-S-yl)-4-aminophenol	120,000
acetaminophen dimer	160,000
2-(N-acetyl-L-cystein-S-yl)hydroquinone	610,000
acetaminophen	740,000
3-hydroxyacetaminophen	1.3×10^6
3-(methylthio)acetanilide	6.9×10^6
N-acetyl-L-cysteine	6.2×10^8
glutathione	2.3×10^9
S-methylglutathione	2.6×10^9
acetaminophen sulfate	$>1.0 \times 10^6$[a]
acetaminophen glucuronide	$>1.0 \times 10^6$[a]
phenacetin	$>1.0 \times 10^6$[a]
aspirin	$>1.0 \times 10^6$[a]

[a]Inhibition was not detected at this concentration.

Effect of Substitution Level

Since our intent was to use the competitive A-B ELISA to quantify 3-Cys-A adducts formed in biological samples at unknown and perhaps variable levels of protein modification, experiments were conducted to determine the effect of adduct substitution level on quantification. Standards of known substitution level were prepared by derivatizing 9,000 *g* liver supernatant with various concentrations of [^{3}H]NAPQI. After extensive dialysis to remove noncovalently bound materials, protein concentrations were determined and the substitution level of each standard was determined by scintillation counting. These synthetic standards, which ranged from 0.5 to 30 nmol 3-(cystein-S-yl)[^{3}H]acetaminophen per mg protein, were analyzed in the competitive immunoassay. When the data were plotted with percent inhibition as a function of protein concentration, the results show an ordered family of inhibition curves where the most highly substituted proteins were the

most efficient inhibitors, and the least substituted protein was the least efficient inhibitor (Figure 5, Panel A). When the same inhibition data were plotted as a function of covalently bound 3-(cystein-S-yl)[^{3}H]acetaminophen, the family of curves were superimposed indicating that under these conditions the competitive A-B ELISA accurately quantifies acetaminophen covalently bound to protein regardless of substitution level (Figure 5, Panel B).

Acetaminophen-induced Hepatotoxicity in Mice

To determine the relationship between the formation of 3-Cys-A adducts in protein and the development of hepatotoxicity, dose response and time course experiments were conducted in male B6C3F1 mice. Using the A-B ELISA specific for 3-Cys-A adducts, we quantified the formation of this adduct in liver and serum protein of mice dosed with acetaminophen. Serum levels of alanine aminotransferase (ALT) were monitored as an index of hepatotoxicity. Administration of acetaminophen at doses of 50, 100, 200, 300, 400, and 500 mg/kg to mice resulted in an increase in serum levels of liver-specific transaminase (evidence of hepatotoxicity) at four hours in the 300, 400, and 500 mg/kg treatment groups only. The formation of 3-Cys-A adducts in liver protein was not observed in the groups receiving 50, 100, and 200 mg/kg doses, but was observed in the groups receiving doses above 300 mg/kg of acetaminophen. Levels of liver adduct were higher in animals receiving the higher doses. 3-Cys-A protein adducts were also observed in serum of mice receiving hepatotoxic doses of acetaminophen. This was an unexpected result. In the time course study, 3-Cys-A adducts in the liver protein reached maximal levels two hours after a 400 mg/kg dose of acetaminophen. By twelve hours the levels decreased to approximately ten percent of the maximal level. In contrast, 3-Cys-A adducts in serum protein were delayed, reaching a sustained maximum six to twelve hours after dosing. The correlation between the appearance of serum aminotransferase and 3-Cys-A adducts in serum protein and the temporal correlation between the decrease in 3-Cys-A adducts in liver protein and the appearance of adducts in serum protein are consistent with a hepatic origin of the 3-Cys-A adducts detected in serum protein (Figure 6). We thus hypothesized that the adducts appearing in serum were of hepatic origin, derived from injured hepatocytes during the development of drug-induced hepatotoxicity and postulated that serum 3-Cys-A protein adducts are a specific biomarker that can be used to study acetaminophen hepatotoxicity (19). Subsequently, the hepatic origin of the 3-Cys-A protein adducts in serum was further confirmed by comparison of adducts detected in SDS-PAGE immunoblots of serum and hepatic protein of B6C3F1 mice at various times after acetaminophen dosing (16). More than 15 proteins containing 3-Cys-A adducts were detected in the liver 10,000 g supernatant. The most prominent protein containing 3-Cys-A adducts in the hepatic 10,000 g supernatant had a relative molecular mass (M_r) of 55 kDa.

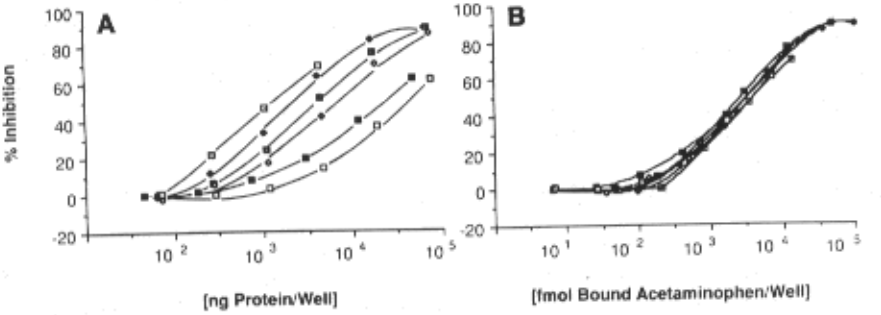


Figure 5. Effect of substitution level on quantitation in the competitive A-B ELISA. The substitution levels based on the nmoles of [3H] NAPQI per mg of protein were (▫) 30, (●) 10, (▪) 5, (◆) 3, (■) 1, (□) 0.5. In panel A acetaminophen-protein adducts were expressed in terms of protein concentration and in panel B, the same inhibition data was plotted as a function of covalently bound acetaminophen equivalents based on radioactivity (Reproduced from Ref. 19).

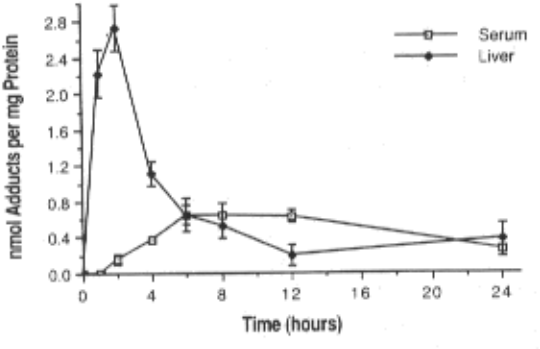


Figure 6. Time course for 3-Cys-A adduct formation in liver and serum proteins. B6C3F1 male mice which had been fasted overnight were administered acetaminophen (400 mg/kg). The procedures for the preparation of the protein samples and the competitive A-B ELISA for quantitation of the acetaminophen are described in (19). The data points are mean ± SEM. All data on acetaminophen adduct formation in serum from 2 to 24 hours are significantly different from control values. The acetaminophen adduct formation in liver protein from one hour to eight hours is significantly different ($P < 0.05$) from control values; however, the acetaminophen adduct formation at the 12 and 24 hour time points are not significantly different from the untreated controls (Reproduced from Ref. 19).

Serum proteins containing 3-Cys-A adducts had molecular masses similar to those found in the liver 10,000 g supernatant (55, 87 and approximately 102 kDa). Collectively, these data indicated that liver adducts were released into the serum following lysis of hepatocytes (16,19).

Detection of Acetaminophen-Protein Adducts in Humans

To determine if acetaminophen toxicity in humans was mediated by a similar mechanism as reported for experimental animals, we looked for the occurrence of 3-Cys-A protein adducts in plasma samples from patients who had taken an overdose of acetaminophen. Plasma was obtained from 30 patients presenting with acetaminophen poisoning at Rigshospitalet, Copenhagen. Plasma samples were stored at -20°C and subsequently shipped frozen to the National Center for Toxicological Research for immunochemical quantification of 3-Cys-A adducts in protein using the competitive PCFIA (20). The patient histories indicated that all patients, except one presenting 58 hours after overdose, were immediately treated with NAC (antidote, NAC) i.v. 300 mg/kg body weight. Plasma obtained at admission was assayed for serum ALT activity and acetaminophen concentration. Of the 30 patients, eleven were at high risk for developing severe liver damage according to Prescott's classification (a nomogram relating risk as a function of time and plasma paracetamol concentration; 21); five patients (Group I) were treated with NAC within 8 hours after overdose, five patients were treated later than

Table II. Concordance between Hepatotoxicity and Acetaminophen-Protein Adducts

Group [n]	I[5]	II[6]	III[3]	IV[16]
Risk Factor#	high	high	moderate	low
Time to NAC* (hours)	4 (1-7)	41 (13-74)	10 (4-15)	10 (2-56)
ALT* (I.U. x 1000)	0.02 (0.01-0.05)	8.4 (4.4-14)	0.04 (0.01-0.08)	0.02 (0.01-0.06)
Plasma 3-Cys-A* (nmol/mg protein)	0	1.9 (0.1-4.1)	0	0▽ (0-0.2)
Plasma acetaminophen*(μM)	1.8 (0.09-2.8)	0.4 (0-1.5)	1.1 (0.2-1.8)	0.6 (0.4-1.3)

Risk of severe liver damage according to Prescot (21).
* Values are the median (range) at admission.
▽ One patient had a value of 0.192.
Adapted from Hinson et al. (20).

8 hours and one was untreated (Group II). Three patients were at moderate risk for developing hepatotoxicity (Group III), and 16 patients were at low risk for developing hepatotoxicity (Group IV) (Table II). All patients that had liver damage, as indicated by elevated plasma ALT, had immunochemically detectable 3-Cys-A adducts in their plasma. These were the patients at high risk of severe liver damage that did not receive antidotal NAC treatment within 8 hours after ingesting acetaminophen (Group II). In contrast, adducts were not found in the plasma of patients who did not show evidence of hepatotoxicity. The relationship between plasma ALT and levels of 3-Cys-A protein adducts at the time of admission is the first direct evidence of a mechanism involving 3-Cys-A adducts in acetaminophen induced liver toxicity in man.

Summary

Immunological approaches were developed to study the the relationship between the binding of acetaminophen to protein and the development of acetaminophen induced hepatotoxicity. Knowledge of the toxic reactive metabolite formed during acetaminophen metabolism and the structure of the resultant 3-Cys-A adduct in protein, suggested the synthesis of a corresponding cysteine-acetaminophen derivative for use as an immunogen. Competitive A-B ELISA and PCFIA were developed and the epitope recognized was extensively characterized. These immunoassays constituted new tools which were used to establish the relationship between the formation of the 3-Cys-A protein adduct in liver and serum and the pathogenesis of acetaminophen toxicity in mice. These tools made it possible to test the hypothesis of an identical mechanism for acetaminophen toxicity in man. Our finding that 3-Cys-A adducts occur in plasma from patients with acetaminophen overdose (20) is preliminary evidence in support of this hypothesis. The close correlation between serum transaminase levels and serum 3-Cys-A adducts is consistent with a hepatic origin for this adduct. In future work this characterized antiserum, coupled with sensitive immunochemical assays and modern protein technology, will provide experimental approaches for the identification and characterization of the protein structures damaged by the acetaminophen metabolite. Such knowledge is needed to understand the processes that ultimately lead to acetaminophen-induced cellular necrosis. This insight is a prerequisite for the development of improved treatment strategies for patients with acetaminophen overdose, and hopefully can be extended to improve understanding of cellular damage from other arylating agents.

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