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ANALYSIS OF ACETAMINOPHEN METABOLITES IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH UV AND AMPEROMETRIC DETECTION

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SUMMARY

Acetaminophen and several of its metabolites are separated isocratically on a reversed-phase (C_{18}) column using a mobile phase of 7% methanol and 0.75% glacial acetic acid in 0.1 *M* KH_2PO_4. Metabolites that can be separated include the sulfate, glucuronide, cysteine, and mercapturic acid conjugates of acetaminophen, as well as 3-hydroxyacetaminophen, 3-methoxyacetaminophen, and 3-methylthioacetaminophen. Although all of the metabolites can be detected by UV spectrophotometry, the sensitivity limits of detection are improved significantly for acetaminophen and all of the metabolites except the sulfate and glucuronide, by amperometric detection (electrochemical) of the same sample as it elutes from the UV detector. Minimal detectable limits (signal-to-noise ratio $\geq$ 2) for acetaminophen and its metabolites, other than the glucuronide and sulfate conjugates, were in the order of 1—2 ng injected on column using UV detection at 248 nm, and 0.1—0.5 ng using electrochemical detection at + 0.60 V with reference to an Ag/AgCl standard electrode.

INTRODUCTION

Acetaminophen is a widely used mild analgesic agent that causes liver necrosis in man and experimental animals when high doses are ingested or administered [1, 2]. Studies on the metabolism of acetaminophen [3, 4] have shown that major routes of elimination involve sulfation and glucuronidation, while a minor route involves oxidation and subsequent conjugation of the oxidation product with the sulfhydryl-containing tripeptide, glutathione (Fig. 1). Evidence strongly implicates a role for this minor oxidation product in the hepatotoxic reaction caused by acetaminophen, although the exact chemical nature of this product and the mechanism(s) by which it leads to cell death are unknown [5, 6].

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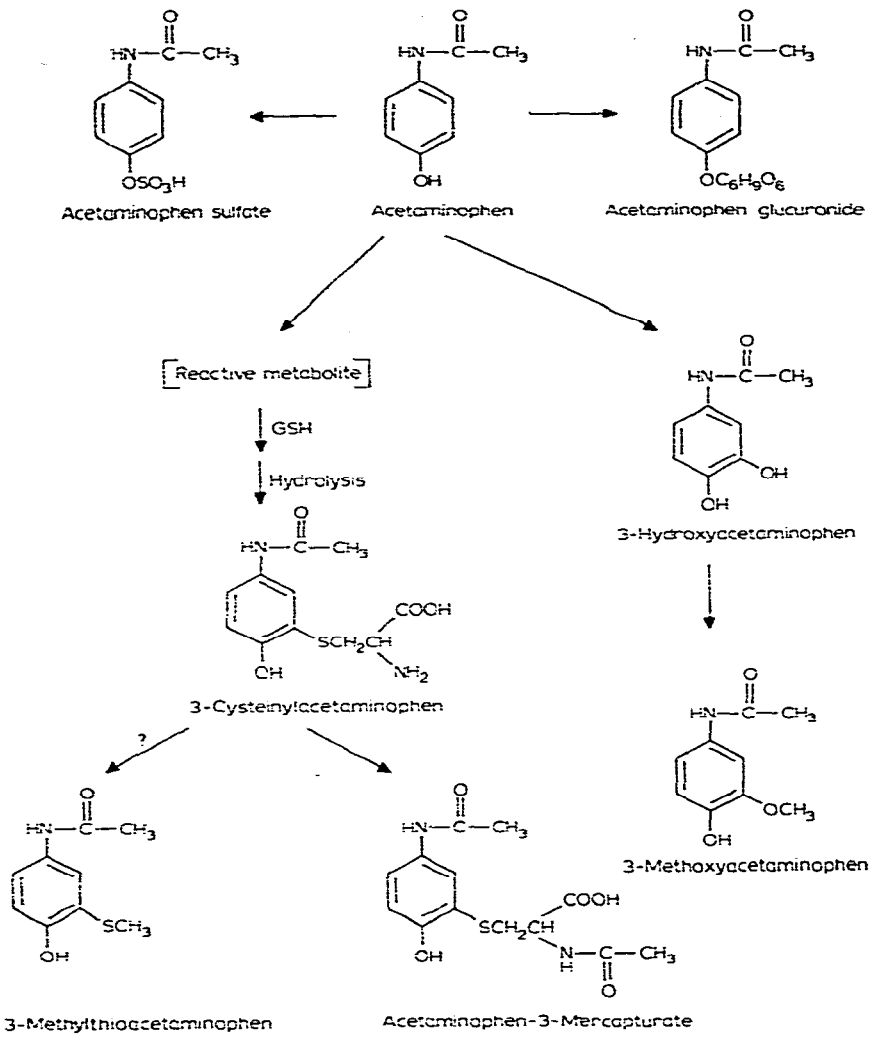

Fig. 1. Partial scheme illustrating the biotransformation of acetaminophen to the metabolites discussed in the text.

In addition to the phenolic glucuronide and sulfate conjugates of acetaminophen, and the cysteine and mercapturic acid conjugates that are derived from the glutathione adduct, several minor metabolites of acetaminophen have been described including catechol and methylated catechols [7] and a methylthio derivative [8]. Knox and Jurand [9] described a high-performance liquid chromatographic (HPLC) method for the separation of acetaminophen and several of its metabolites, and they reported that 3-methoxyacetaminophen was a major metabolite in urine from patients who took overdoses of acetaminophen [10]. This observation may be significant in that both 3-hydroxyacetaminophen and 3-methoxyacetaminophen have been found to be hepatotoxic [11, 12].

Although several methods have been described for monitoring acetaminophen and its metabolites by HPLC with UV detection [9, 13—16], none has

attempted to assay all of the metabolites that have been described. Miner and Kissinger [17, 18] have detailed the advantages of using HPLC with amperometric (electrochemical) detection for the analysis of acetaminophen and some of its sulfur ether conjugates. This method was successfully applied to a study on acetaminophen bioavailability by Munson et al. [19].

In this report we describe an assay that separates the known metabolites of acetaminophen and utilizes tandem UV and electrochemical detection for improved selectivity and sensitivity.

MATERIALS AND METHODS

Apparatus

Chromatography was performed on a component system consisting of a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A solvent delivery system and Model U6K injector with a 2-ml sample loop. Separations were carried out on a 30 cm × 4.6 mm I.D. μBondapak C_{18} (10 μm particle size) column (Waters Assoc.). The mobile phase was 7% methanol and 0.75% glacial acetic acid in 0.1 *M* KH_2PO_4. All water was Milli-Q grade (Millipore, Bedford, MA, U.S.A.). Typically, a flow-rate of 2 ml/min at 1650 p.s.i. was employed. Flow was directed through a Model 450 variable-wavelength UV absorbance detector (Waters Assoc.) output to a Linear Precision Model 261 strip chart recorder (Linear Precision, Irvine, CA, U.S.A.), in tandem with an LC-4 amperometric detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.) output to a Model 3385 recording integrator (Hewlett-Packard, Avondale, PA, U.S.A.).

The variable-wavelength detector was set at 248 nm, the absorption maximum for acetaminophen. Typical detector attenuation was 1.0 a.u.f.s. The amperometric detector consisted of an RC-1 reference compartment and a TL-3 glassy carbon electrode referenced to an Ag/AgCl electrode. For most assays the working potential was set at + 0.60 V over the oxidizing electrode. Electrometer output was varied from 10—50 nA/V.

Materials

Acetaminophen was purchased from Eastman Organic Chemicals (Rochester, NY, U.S.A.). Acetaminophen glucuronide was prepared by the method of Shibasaki et al. [20], and acetaminophen sulfate by the method of Smith and Timbrell [21]. 3-Cysteinylacetaminophen and 3-methylthioacetaminophen were synthesized as described by Focella et al. [22]. Acetaminophen-3-mercapturate was prepared by the acetylation of 3-cysteinylacetaminophen. This method gives low yields of the desired mercapturate which must be purified by preparative HPLC using conditions previously described [15]. All of the synthetic metabolites had melting points similar to literature values and mass spectral characteristics consistent with their structures [23].

3-Hydroxyacetaminophen (3′,4′-dihydroxyacetanilide) was synthesized according to the method of Szent-Gyorgyi et al. [24]. 3-Methoxyacetaminophen (3′-methoxy-4′-hydroxyacetanilide) was prepared in a similar reductive acetylation reaction starting with 4-nitroguaiacol. Spectral data [NMR and high-resolution mass spectrometry (MS)] were consistent with the structure. Details will be reported elsewhere.

Procedures

Urine samples were processed by measuring the total urine volume and then by transferring 1-ml portions of the urine to two vials containing 4 ml each of 2 *M* acetate buffer, pH 5.0. To one vial was added 50 μl of β-glucuronidase—sulfatase (Sigma, St. Louis, MO, U.S.A.). This sample was incubated overnight at 37°C to hydrolyze conjugates. Portions (200 μl) of each sample were then added to a polyethylene microcentrifuge tube and spun down for 5 min at maximum speed in a Beckman Model 152 microcentrifuge to separate the suspended material. Usable column life under the stated conditions and with injections of 10—20 μl of the diluted urine samples was approximately 500 h. Although k' was significantly improved for all metabolites relative to acetaminophen by using an Ultrasphere ODS-5 column (Altex, Arlington Heights, IL, U.S.A.), we have not yet fully evaluated this column with regards to column life.

RESULTS

The structures of acetaminophen and those of its metabolites that were analyzed by the HPLC method are illustrated in Fig. 1. Because of limited quantities of some of the metabolites, standard solutions were prepared in water and stored in a freezer. Dilutions were then made as needed. The catechol and cysteine metabolites were found to be unstable even when frozen, therefore standard solutions of these compounds were prepared for each daily run.

Voltammograms

Normalized peak area—potential curves for acetaminophen and its metab-

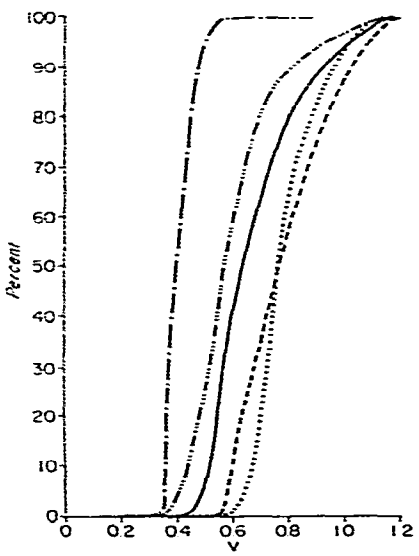

Fig. 2. Normalized peak area—potential curves for acetaminophen (· · ·), 3-hydroxyacetaminophen (— · · · —), 3-methoxyacetaminophen (———), 3-cysteinylacetaminophen (— — —), and 3-methylthioacetaminophen (— · —).

olites were generated by injecting 20 ng of each standard in 10 μl of water onto the chromatography system with the detector set at several electrode potentials and recording peak areas (Fig. 2). Approximate maximal peak currents for each of the compounds were 390 nA (acetaminophen), 175 nA (3-methoxyacetaminophen), 110 nA (3-hydroxyacetaminophen), 45 nA (3-cysteinylacetaminophen), and 40 nA (3-methylthioacetaminophen). Because of the limited quantity of acetaminophen mercapturate that was available, a curve was not generated for this metabolite, although its electrochemical response was found to be very similar to the cysteine conjugate at the working potential used in our assays. An electrode potential of + 0.60 V was chosen for most assay work to maximize the response of the catechol metabolite, 3-hydroxyacetaminophen, and to minimize interference from endogenous urinary components.

Linearity studies

Linearity was evaluated with eight standard solutions containing varying amounts of acetaminophen and its metabolites to produce the concentration ranges described in Table I. Either peak heights or peak areas gave linear curves; results are reported for peak height determinations.

Standard curves were fitted by computer assisted regression analysis and the correlation coefficients were $\geqslant 0.997$ for all compounds detected by UV and $\geqslant 0.996$ for all compounds detected by electron-capture. Reproducibility was

TABLE I

ACCURACY AND SENSITIVITY OF THE METHOD

Compound	Concentration range of standard curve (μg/ml)	Relative standard deviation* (%)		Sensitivity** (ng/ml)		Sensitivity in urine diluted 1:4*** (ng/ml)	
		UV	EC	UV	EC	UV	EC
Acetaminophen	0.42—42	± 2.6 (4.5)	± 4.7 (4.5)	70	15	500	60
Acetaminophen glucuronide	1 —500	± 2.2 (5.4)	—	35	—	500	—
Acetaminophen sulfate	0.45—230	± 2.3 (4.7)	—	45	—	1000	—
3-Hydroxyacetaminophen	0.37—37	± 4.9 (3.1)	± 5.5 (3.1)	150	15	1200	500
3-Methoxyacetaminophen	0.5 —100	± 3.5 (2.2)	± 2.2 (11.2)	100	10	200	20
3-Cysteinylacetaminophen	0.35—35	± 3.9 (1.7)	± 9.7 (1.7)	250	50	2400	300
3-Methylthioacetaminophen	0.53—70	± 2.3 (3.1)	± 2.6 (3.1)	117	22	2800	300

*Relative standard deviations were determined at the concentrations (μg/ml) indicated in parentheses. Twenty injections of 10 μl each were made for all compounds in the UV precision study and ten injections for all compounds in the electrochemical precision study.

**Sensitivities were estimated with reference to a signal-to-noise ratio of about 2, using 10-μl injections of water to which pure standards had been added.

***Sensitivities were estimated with reference to a signal-to-noise ratio of about 2, using 10-μl injections of pooled human urine to which pure standards had been added.

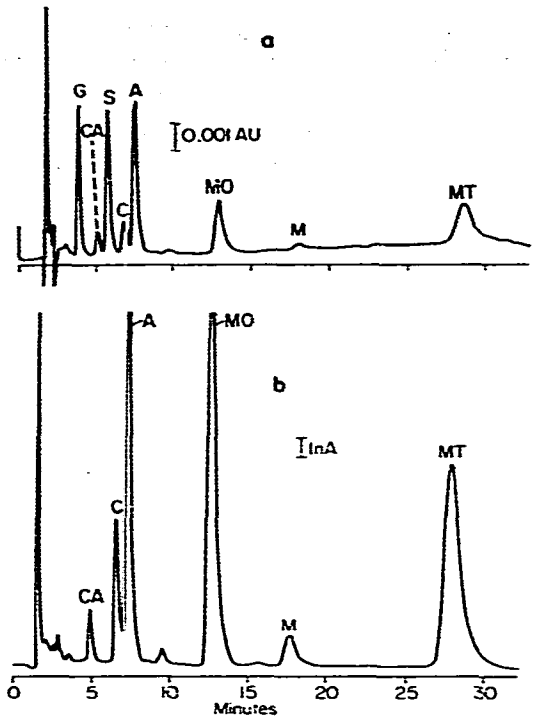

Fig. 3. Chromatograms of a standard solution that contained acetaminophen glucuronide (G, 5.4 μg/ml), the catechol 3-hydroxyacetaminophen (CA, 3.1 μg/ml), acetaminophen sulfate (S, 4.7 μg/ml), 3-cysteinylacetaminophen (C, 1.7 μg/ml), acetaminophen (A, 4.5 μg/ml), 3-methoxyacetaminophen (MO, 2.2 μg/ml), acetaminophen-3-mercapturate (M, 1.5 μg/ml), and 3-methylthioacetaminophen (MT, 5 μg/ml). (a) With UV detection at 248 nm and (b) with electrochemical detection at + 0.60 V.

determined by multiple injections of acetaminophen and its metabolites as described in Table I. Relative standard deviations are given for concentrations that were low on the standard curves; at higher concentrations the precision improved, particularly with electrochemical detection. Minimum detectable concentrations for each compound are provided as estimates of sensitivity (Table I). Thus, in the absence of a urine matrix, a minimum of 700 pg of acetaminophen could be detected by UV and a minimum of 150 pg could be detected by electron-capture (signal-to-noise ratio = 2). Determinations were made by injecting 10 μl of standard mixtures in water into the 2-ml sample loop and relating peak heights to the noise levels. Although linearity curves could not be generated for acetaminophen-3-mercapturate because of a lack of sufficient standard, injections of a few concentrations indicated linearity of detector response as well.

Typical chromatograms from single 10-μl injections of a standard solution of acetaminophen and its metabolites are presented in Fig. 3a and b. The chromatogram that was obtained by monitoring the HPLC effluent with a UV detector (Fig. 3a) shows all of the metabolites for which we had standards. The chromatogram that was obtained by monitoring the same effluent with the electrochemical detector (Fig. 3b) shows the expected enhanced response for acetaminophen and metabolites other than the glucuronide and sulfate conjugates.

Studies in urine

Recovery studies were carried out in urine obtained from subjects who were not taking drugs, by adding stock standard solutions of acetaminophen and its metabolites to the urine in order to achieve concentrations used in the linearity studies. Recoveries were calculated by comparing peak heights that were generated from equivolume injections of the matrix-free standards. Recovery for all compounds was nearly 100% at all dilutions. However, minimum detectable concentrations for acetaminophen and its metabolites were greater when they were measured in urine samples (Table I), presumably because of

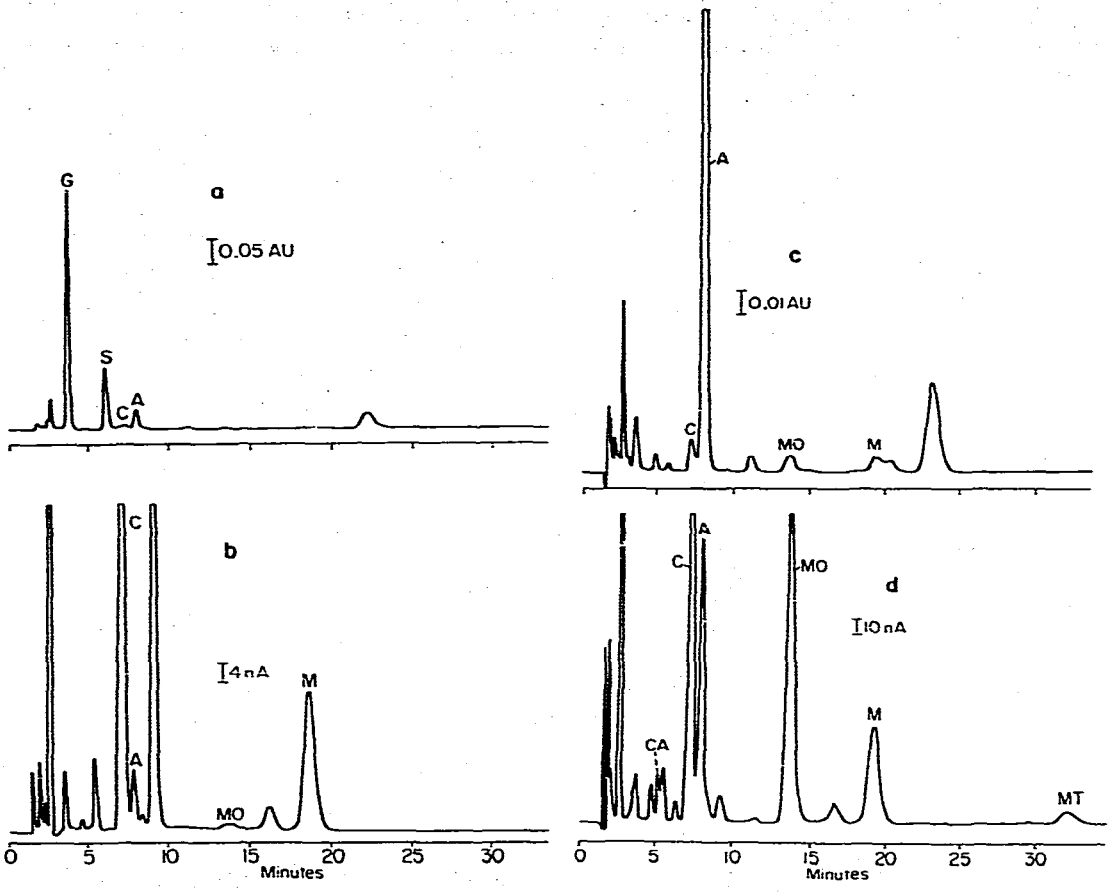

Fig. 4. (a) Chromatogram (UV detection, 248 nm) of a 10-µl sample of a urine aliquot diluted 1:4 with 2 *M* acetate buffer, pH 5.0. Based on standard curves and recovery data, we estimated the amounts of acetaminophen glucuronide (G), acetaminophen sulfate (S), and free acetaminophen (A) to be 304 µg/ml, 112 µg/ml, and 23 µg/ml, respectively. (b) Chromatogram (electrochemical detection, + 0.55 V) of the same eluent indicating the presence of the cysteine conjugate (C), free acetaminophen (A), free methoxyacetaminophen (MO) and acetaminophen-3-mercapturate (M). (c) Chromatogram (UV detection, 248 nm) of a 10-µl sample of a second aliquot of the patient's urine after β-glucuronidase—sulfatase hydrolysis. (d) Chromatogram (electrochemical detection, + 0.55 V) of the same eluent. Based on standard curves and recovery data, this hydrolyzed sample contained 3-hydroxyacetaminophen (CA, 0.43 µg/ml), 3-cysteinylacetaminophen (C, 30 µg/ml), acetaminophen (A, > 400 µg/ml), 3-methoxyacetaminophen (MO, 78 µg/ml), acetaminophen-3-mercapturate (M, ca. 91 µg/ml), and 3-methylthioacetaminophen (MT, 11 µg/ml).

noise generated by constituents that were normally present. Some known constituents such as caffeine and theobromine were found to have retention times almost coincident with 3-methylthioacetaminophen and acetaminophen-3-mercapturate, respectively, and do interfere with normal UV detection. Fortunately, these constituents do not react electrochemically at the potentials that were used for monitoring the acetaminophen metabolites. Unknown constituents that absorb in the UV were found to have retention times virtually identical to the catechol metabolite, 3-hydroxyacetaminophen, as well. Unfortunately some constituents of urine that nearly coelute with the catechol are also electrochemically active, and therefore they increase the minimum detectable limit for this metabolite to a level similar to that found in most urine samples (ca. 0.5 μg/ml).

Chromatograms of an unhydrolyzed and hydrolyzed (β-glucuronidase—sulfatase) urine sample (diluted 1:4 with 2 *M* acetate buffer, pH 5.0) that was obtained from a patient who had ingested an unknown quantity of acetaminophen, are presented in Fig. 4a—d. The spectrum that was recorded for an unhydrolyzed sample (UV detection, Fig. 4a) shows relatively large quantities of the glucuronide and sulfate conjugates. Electrochemical detection of the same unhydrolyzed sample (Fig. 4b) revealed the presence of the catechol, cysteine, methoxy and mercapturic acid metabolites of acetaminophen. After enzymatic hydrolysis of the urine the spectrum that was recorded by UV detection (Fig. 4c) showed significantly larger quantities of acetaminophen and its catechol, cysteine, methoxy and mercapturic acid metabolites. The increase in free acetaminophen levels is particularly striking, as would be expected from hydrolysis of the glucuronide and sulfate conjugates which are the major metabolites of acetaminophen. Finally, electrochemical detection of this sample (Fig. 4d) showed two especially interesting features: (1) substantially increased sensitivity of detection by the electrochemical technique for 3-methoxyacetaminophen, and (2) substantially increased sensitivity of detection for 3-methylthioacetaminophen, a metabolite that was undetectable in all of the previous chromatograms. Slight differences in metabolite retention times compared to the standard chromatograms (Fig. 3a, b) are a reflection of the newer column used in this study.

DISCUSSION

A multi-faceted approach to the analysis of acetaminophen and its metabolites has been presented. The method is applicable to metabolism and pharmacokinetic studies of acetaminophen disposition in patients taking both normal and overdoses of the drug (work in progress). The method involves the direct analysis of diluted urine samples before and after enzymatic hydrolysis of conjugates. The assay utilizes HPLC on a single reversed-phase column that is eluted with an acidic buffer to separate the metabolites, and the detection of metabolites in the eluent by both UV and electrochemical methods.

Acetaminophen glucuronide and sulfate are most easily measured in 1:20 to 1:50 dilutions by UV detection. All other metabolites are most selectively analyzed by electrochemical detection as free compounds in urine that has been diluted 1:4. We realize that many of these metabolites may exist as

glucuronide and/or sulfate conjugates; however, at the present time we do not have standards of these possible conjugated metabolites.

The primary advantages of this method over existing HPLC methods for quantifying acetaminophen and its metabolites are (1) the ability to monitor levels of catechol metabolites of acetaminophen (3-hydroxy- and 3-methoxy-acetaminophen) and 3-methylthioacetaminophen, all of which may play a role in acetaminophen-induced toxicities, and (2) improved selectivity and sensitivity, by using electrochemical detection, for acetaminophen and its metabolites other than the sulfate and glucuronide. Work is now in progress to improve the precision of the assay by employing an internal standard, 2-hydroxyacetanilide, which can be detected both spectrophotometrically and amperometrically.

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