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UTILITY OF SOLUTION ELECTROCHEMISTRY MASS SPECTROMETRY FOR INVESTIGATING THE FORMATION AND DETECTION OF BIOLOGICALLY IMPORTANT CONJUGATES OF ACETAMINOPHEN

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SUMMARY

On-line formation and detection of glutathione and cysteine conjugates of acetaminophen were accomplished by the interfacing of a coulometric electrochemical cell with a thermospray mass spectrometer in a flow-injection experiment using a liquid chromatographic pump. Formation of the conjugates occurred only after acetaminophen was oxidized electrochemically by a two-electron transfer to N-acetyl-*p*-benzoquinoneimine and reacted in a mixing tee with either glutathione or cysteine. The newly formed conjugate was detected by thermospray mass spectrometry by observing the $[M + H]^+$ ion for the acetaminophen–glutathione conjugate at *m/z* 457, or the $[M + H]^+$ ion for the acetaminophen–cysteine conjugate at *m/z* 271. Both the glutathione and cysteine conjugates produced a common fragment ion at *m/z* 184. The on-line reaction of glutathione and electrochemically generated N-acetyl-*p*-benzoquinoneimine was monitored at varying pH. At pH 8.5 the ion intensity for the acetaminophen–glutathione conjugate was greater than at lower pH, indicating that lower proton concentration enhanced the reaction of glutathione with N-acetyl-*p*-benzoquinoneimine. This on-line electrochemical–thermospray mass spectrometric method demonstrated that acetaminophen conjugates may be formed and detected in the time frame of 1 s.

INTRODUCTION

Thermospray mass spectrometry (TSP MS) has been an indisputable success as an interface and detection mode for high-performance liquid chromatography (HPLC) and as a soft ionization mode for non-volatile compounds. A good part of this success stems from the fact that the mobile phases used in reversed-phase HPLC, the method of choice for many of today's analyses by HPLC, usually utilize polar solvents and various salts that buffer the mobile phase. These types of polar mobile phase for reversed-phase HPLC are similar to the liquid carriers best suited for maximizing the thermospray effect. This excellent match between suitable operating conditions for reversed-phase HPLC and TSP MS is fortuitous for the viable interfacing of these two methods.

The utilization of polar solvents with various salts and buffers also allows in-

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terfacing of electrochemical methods with TSP MS. Because TSP MS is normally operated at flow-rates of 1–2 ml min^{-1}, this makes the coupling of electrochemistry (EC) with TSP MS very simple and convenient. This flow-rate also permits the thermospray mass spectrometer to be employed in the flow-injection mode. The initial development of the EC–TSP MS interface was started by Hambitzer and Heitbaum[1], and by one of the authors of this study in Marvin Vestal's laboratory[2]. Later work by Volk *et al.*[3] investigated the electrochemical oxidation of purines by tandem mass spectrometry (MS–MS) and demonstrated the utility of EC–TSP MS for studying short-lived electrochemically generated intermediates. Previous to the development of TSP MS[4], electrochemical methods were coupled to MS for purposes of detecting electrolysis products[5,6], but these products had to be gaseous in order to accommodate the mass spectrometer's vacuum environment. Only with the advent of TSP MS and its flow-injection capabilities did the EC–MS interface for directly studying non-volatile compounds in solution on-line become feasible. In order to differentiate the EC–TSP MS interface from the EC–MS interface, which detects only volatile products, the name "solution electrochemistry mass spectrometry" (SECYMS) was given to this EC–TSP MS interface[2].

The conjugation of glutathione (GSH) to acetaminophen (APAP) has been reported by Potter and Hinson[7] to go through a two-electron oxidation step for APAP followed by reaction with GSH. Recently the enzymic reaction of the oxidized product of APAP, *i.e.* N-acetyl-*p*-benzoquinoneimine (NAPQI), and its spontaneous reaction with GSH were investigated by stopped-flow experiments[8]. The direct analysis by TSP MS of rat bile for APAP conjugates by Betowski *et al.*[9] demonstrated that the GSH conjugate may be detected as the $[M+H]^+$ ion.

As an extension of the SECYMS method, the electrochemical oxidation of APAP to NAPQI was performed on-line as a constant flow-injection experiment followed by TSP MS. After injection of GSH into the stream of NAPQI, the reaction was monitored by the thermospray mass spectrometer. Preliminary results showed that this is a very rapid (*ca.* 1 s) and simple method for forming and detecting the acetaminophen–glutathione (APAP-SG) conjugate[10]. Details of the APAP-SG formation, as well as similar experiments with APAP and cysteine (CSH) conjugation to form the acetaminophen–cysteine (APAP-SC) conjugate are reported in this paper.

EXPERIMENTAL

A diagram of the flow-injection system for forming and detecting conjugates of APAP by SECYMS is shown in Fig. 1. For the APAP-SG formation, a $3.3 \cdot 10^{-5}$ *M* APAP solution in water–methanol (90:10) containing 0.1 *M* ammonium acetate was placed in the syringe pump (ISCO LC-5000). The reciprocal pump (Waters 6000A) contained an identical solution, but without APAP. The solution containing APAP was pumped through an electrochemical coulometric guard cell (ESA, Model 5020) at various potentials as maintained by a potentiostat (ESA, Model 5100A). The potential was reported as referenced to a palladium reference electrode[11]. The potentiostat was modified so that guard cell currents could be fed to an integrator. Solutions of GSH diluted in water–methanol (90:10) containing 0.1 *M* ammonium acetate were injected into the stream of APAP by an HPLC injector (Rheodyne, Model 7125) with a 100-μl loop and reacted in a stainless-steel mixing tee with a 0.043 in. through-hole

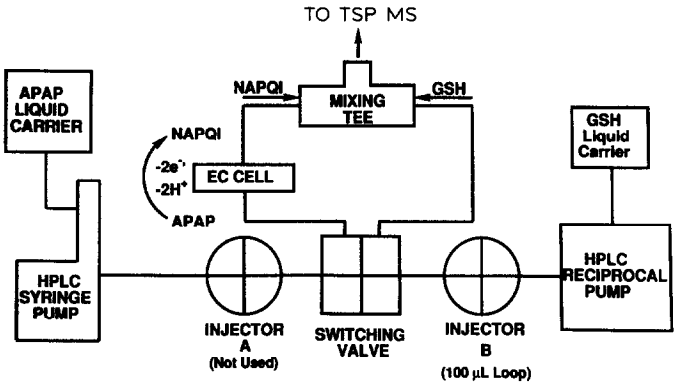

Fig. 1. Flow-injection system used to form and detect acetaminophen–glutathione conjugates by TSP MS. For the case of the cysteine conjugate, the acetaminophen solution was in the reciprocal pump and the 100-μl loop on injector A.

(Scientific Systems Inc.). The mixed streams of APAP and GSH were then directly pumped into a quadrupole mass spectrometer (Delsi R-1010C) equipped with a thermospray probe (Vestec) and thermospray source (Delsi). The vaporizer and block temperatures were maintained by a thermospray controller (Vestec). Solutions of different pH were made of ammonium acetate aqueous solutions adjusted with acetic acid for pH below neutral and ammonium hydroxide for pH > 7. For each pH evaluated, the pH for the GSH and APAP solutions, and the pH for the liquid carrier for injections were equivalent. Potentials were manually adjusted on the potentiostat (non-scanning mode). Temperature conditions for TSP MS were: vaporizer (T_1), 137°C; tip (T_2), 246°C; and block 285°C. The flow-rates for syringe and reciprocal pumps were 0.7 ml min^{-1} each; the total flow into mass spectrometer was 1.4 ml min^{-1}.

The procedure for forming CSH conjugates was identical with that used for APAP-SG, except the APAP-containing solutions were pumped by the reciprocal pump. A solution of $1.8 \cdot 10^{-4}$ *M* APAP was used to form the CSH conjugate. The concentration of cysteine injected was 1 mg ml^{-1} in a 100-μl loop. Thermospray conditions were: vaporizer (T_1), 140°C; tip (T_2), 198°C; and block, *ca.* 250°C. Flow-rates for the reciprocal and syringe pump were 0.5 ml min^{-1} each for the APAP-SC.

Cyclic voltammetry and differential pulse voltammetry utilized a BAS (Bioanalytical Systems) glassy carbon electrode as the working electrode. Waveforms were generated by a BAS-100A electroanalytical system. In both cases, potentials were referenced to an Ag/AgCl electrode, and a platinum wire was the counter electrode. The electrolyte was 0.1 *M* ammonium acetate (pH 4.5) for the APAP and APAP-SG cyclic voltammetry. The cyclic and differential pulse voltammetry study of GSH and CSH employed 0.1 *M* ammonium acetate (pH 7) as electrolyte. Differential pulse voltammetry was carried out at a scan-rate of 5 mV s^{-1} with a pulse amplitude of 50 mV. Solute concentrations were *ca.* $1 \cdot 10^{-3}$ *M*.

An authentic sample of APAP-SC was synthesized using methods similar to those previously described[7,12]. The identity of the conjugate was proven by TSP MS and proton NMR spectrometry, and the purity of the conjugate was evaluated by analytical HPLC with UV detection and with electrochemical detection. Electro-

chemical detection [13] indicated that the APAP-SC contained 4.1% cysteine. The analysis of APAP-SC by TSP MS was performed in 0.1 *M* ammonium acetate at a flow-rate of 1.0 ml min^{-1} without a chromatographic column.

All mass spectral scans in this study by TSP MS were full scan range (*ca.* 100–500 *m/z*). The term "selective ion monitoring" is used in this text to refer to the selected m/z plots after a full scan measurement.

RESULTS AND DISCUSSION

The reported electrochemical oxidation of APAP in water, which is catalyzed by H^+, is shown in Fig. 2, pathway A[14]. Cyclic voltammetry of APAP at a scan-rate of 50 mV s^{-1} gave an oxidation wave, which is indicative of the electrochemical conversion to NAPQI, at a potential of +0.58 V *vs.* Ag/AgCl. A quasireversible wave for the reduction of NAPQI to APAP was observed at *ca.* −0.08 V *vs.* Ag/AgCl. When GSH was added to APAP in a 1:1 mole ratio, the cyclic voltammogram showed an oxidation wave at +0.60 V *vs.* Ag/AgCl, but no quasireversible wave for the reduction of NAPQI back to APAP. This indicated that GSH reacted completely with the electrochemically generated NAPQI at the electrode interface and this led to no observance of the reduction of NAPQI. This reaction of NAPQI with GSH is shown in Fig. 2, pathway B.

The electrochemical study of APAP is a good candidate for the technique of SECYMS because of the electrochemical quasireversible nature of APAP[15]. As shown in Fig. 3a, when analyzed by TSP MS, APAP produced an $[M+H]^+$ ion at *m/z* 152, an $[M+NH_4]^+$ ion at *m/z* 169, and an $[M_2H]^+$ ion at *m/z* 303. Upon oxidation of APAP to NAPQI utilizing the on-line electrochemical coulometric ESA guard cell, NAPQI was detected at an applied oxidation potential of *ca.* +0.5 V. As shown in Fig. 3b, NAPQI produced an $[M+H]^+$ ion at *m/z* 150 and a fragment ion at *m/z* 108, which may be due to protonated *p*-benzoquinoneimine formed from deacetylation of APAP. A residual mass spectral peak at *m/z* 152 for the $[M+H]^+$ ion of APAP is also observed. This residual APAP $[M+H]^+$ ion is due to the electrochemical quasireversible nature of APAP[15].

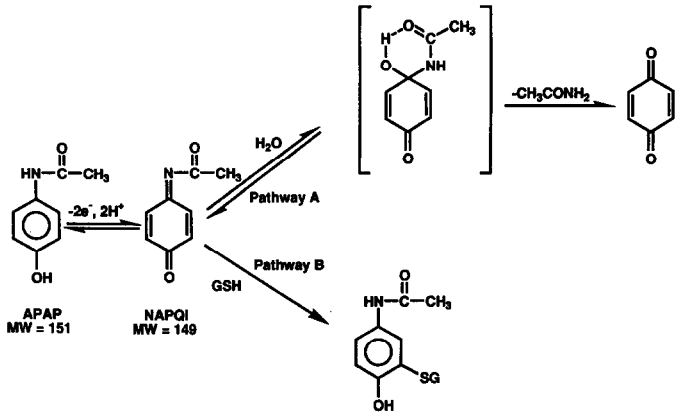

Fig. 2. Electrochemical oxidation of APAP as it occurs by pathway A in H_2O as catalyzed in acidic media (ref. 14) and by pathway B with glutathione.

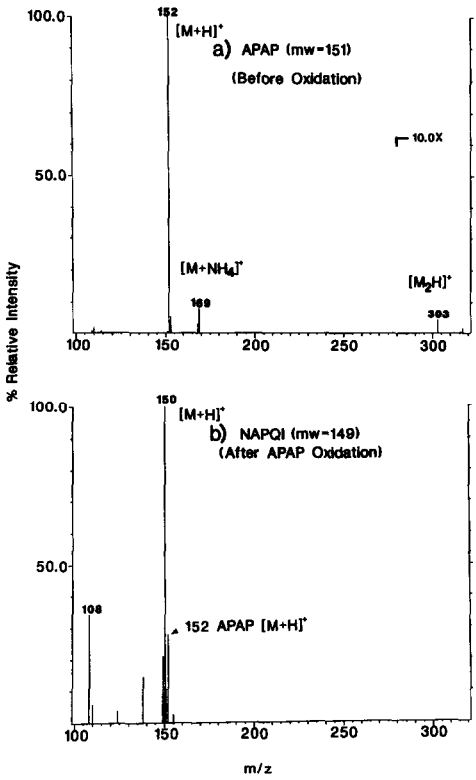

Fig. 3. TSMS of APAP in 0.1 *M* ammonium acetate obtained (a) before oxidation of APAP to NAPQI and (b) after oxidation of APAP to NAPQI at *ca.* +0.5 V. Mass spectral peaks at *m/z* 150 and 108 are indicative of NAPQI.

The TSP MS of APAP-SG has been reported, the $[M+H]^+$ ion at *m/z* 457 and a fragment ion at *m/z* 184 were observed[9]. The fragment ion corresponded to a cleavage of the GSH molecule from the APAP, leaving only a thiol attached to the APAP moiety. The thiol fragment, APAP-SH, had a much higher ion intensity than the peak intensity for the $[M+H]^+$ ion for APAP-SG.

The flow-injection arrangement in Fig. 1 was utilized to form and detect the APAP-SG conjugate on-line. At a constant flow-rate of APAP through the electrochemical cell (labeled EC cell in Fig. 1), the potential was increased by 0.05 V increments until APAP was oxidized to NAPQI. Complete oxidation of APAP in the coulometric electrochemical cell occurred at +0.2 V, as indicated by the hydrodynamic voltammogram in Fig. 4. The oxidation of APAP to NAPQI was confirmed by TSP MS. A 100-μl volume of the GSH solution was injected into the stream of NAPQI and it reacted in less than 1 s to form the APAP-SG conjugate. The time of formation was calculated from the volume inside the stainless-steel tubing after the mixing tee, and the flow-rate. The results of this reaction are illustrated in Fig. 5. Before oxidation of APAP, injection of the GSH solution produced only the $[M+H]^+$ ion for GSH at *m/z* 308 and fragment ions for GSH at *m/z* 179, 147, and 130 (Fig. 5a). When APAP was oxidized to NAPQI, and then mixed with GSH, the

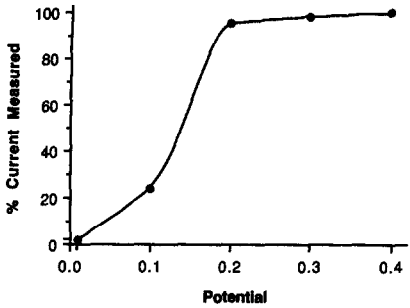

Fig. 4. Hydrodynamic voltammogram of APAP obtained by constant flow of APAP in water–methanol (90:10) containing 0.1 *M* ammonium acetate through the coulometric ESA guard cell. The potential is given in volts *vs.* reference.

APAP-SG conjugate is detected by TSP MS at *m/z* 457 for the $[M+H]^+$ ion and the corresponding fragment ion at *m/z* 184 (Fig. 5b).

The constant flow of APAP was monitored on-line by TSMS at various applied potentials. The selective ion monitoring scan of *m/z* 150 and 108, ions indicative of

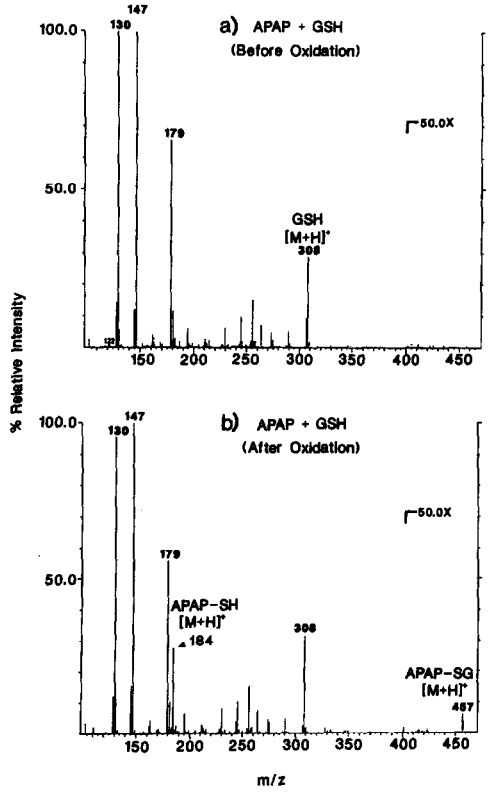

Fig. 5. TSP MS of APAP mixed with GSH acquired (a) before oxidation of APAP and (b) after oxidation of APAP. After oxidation of APAP, mass spectral peaks indicative of APAP-SG are seen for $[M+H]^+$ at *m/z* 457 and the protonated thiol fragment ion at *m/z* 184.

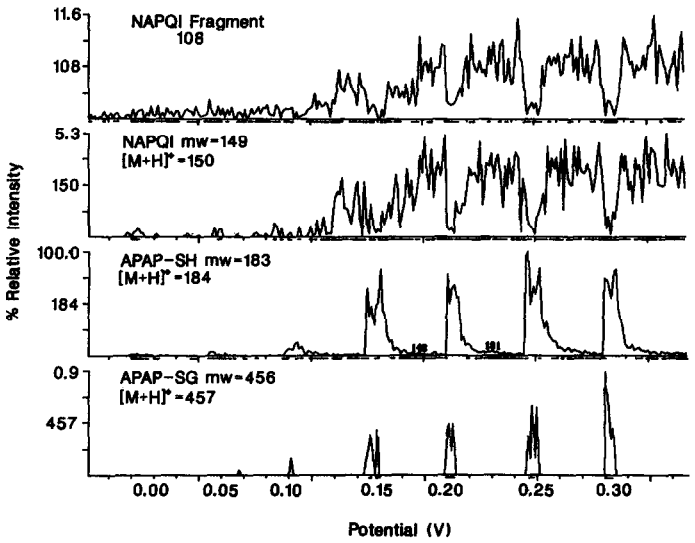

Fig. 6. Selective ion monitoring of ions indicative of NAPQI (*m/z* 150 and 108) and APAP-SG (*m/z* 457 and 184) shown at various applied potentials. Note that ions associated with APAP-SG did not appear until NAPQI was formed.

the oxidation of APAP to NAPQI, is seen in Fig. 6 as a function of potential. Initially, only background ion intensity was observed for the monitoring of *m/z* 150 and 108, but when NAPQI began to form these ion intensities increased significantly. This occurred at *ca.* +0.2 V. A point to remember is that the stream of APAP is continuously being oxidized to NAPQI at potentials greater than +0.2 V, thus the selective ion scans for *m/z* 150 and 108 appeared as a plateau above +0.2 V, and only background intensity is observed at non-oxidizing potentials. GSH was injected into this stream of constantly formed NAPQI. These injections corresponded to peaks observed in Fig. 6 for scans of *m/z* 457 and 184, ions representing the APAP-SG conjugate. A feature to note from Fig. 6 is that the conjugate was observed only after APAP was oxidized to NAPQI. The emergence of ion intensities for monitoring *m/z* 457 and 184 correlated with the growth of ion intensities for the NAPQI ions. Another feature to note is that upon injection of GSH, the peaks observed for the ions due to formation of APAP-SG correspond to the valleys in the selective ion scans for the NAPQI fragment. This feature is expected because the NAPQI is being depleted by the reaction with GSH to form the APAP-SG conjugate.

The formation of APAP-SG was evaluated at varying pH *vs.* peak area of the ion intensities. The ion intensities for the ions corresponding to APAP-SG, formed after the on-line electrolysis of APAP and injection of GSH, were determined as a function of pH as shown in Fig. 7. At a pH 8.5, the ion intensity for APAP-SG is considerably higher than at pH 4.5. It may be interpreted from Fig. 2 that at lower pH the alternative pathway for the oxidation of APAP to NAPQI, which is catalyzed by H^+ [14], to form 1,4-benzoquinone (pathway A) is competing with the GSH conjugation (pathway B). At higher proton concentrations pathway A becomes more prominent than pathway B, hence the decrease in intensities for the APAP-SG conjugate.

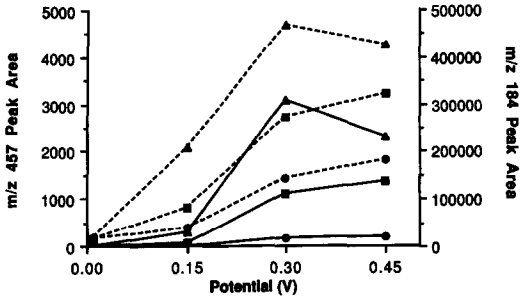

Fig. 7. Peak area of ion intensities for APAP-SG ions at *m/z* 457 (——) and 184 (---) formed and detected at various potentials and pH. (●) pH 4.5; (■) pH 6.0; (▲) pH 8.5.

The pH affects the potential where APAP begins to oxidize to NAPQI. At lower pH, the oxidation is more difficult, *i.e.* a higher positive potential is needed to convert APAP into NAPQI. This pH effect on oxidation has been reported for APAP[16]. Hydrodynamic voltammograms in the ESA coulometric electrochemical cell confirmed this effect of pH. This is indicated in Fig. 7 by the intensity of *m/z* 457 at pH 4.5 and pH 8.5. For the potential of +0.15 V, the lower pH of 4.5 produced no ion intensity for the $[M+H]^+$ ion compared with the $[M+H]^+$ ion intensity at pH 8.5 for +0.15 V.

The electrochemical cell employed in this study was a coulometric cell. If the concentration of a constant flow of solute through the electrochemical cell is known, then the number of electrons transferred during the redox event may be calculated by

$$i = (n\,F\,f\,C)/(60\text{ s min}^{-1})$$

where i is the constant current that is measured, n is the number of electrons transferred, F is Faraday's constant (96 500 A s mol^{-1}), f is the flow-rate in ml min^{-1}, and C is concentration of solute in mol ml^{-1}. For the case of APAP, the expected two-electron transfer was confirmed by the flow coulometric measurement.

The thermospray mass spectrum of an authentic sample of the cysteine conjugate of APAP is shown in Fig. 8. The $[M+H]^+$ ion for APAP-SC at *m/z* 271 and the protonated APAP-SH fragment ion at *m/z* 184 were observed. The ammoniated form of the fragment ion was also detected at *m/z* 201.

The APAP-SC conjugate was also electrochemically formed on-line and detected by TSP MS in a similar manner to the APAP-SG conjugate. The TSP MS of APAP before oxidation and mixing with CSH is shown in Fig. 9a. Mass spectral peaks for CSH were detected for the $[M+H]^+$ ion at *m/z* 122 and the $[M_2H]^+$ ion at *m/z* 243. Two undetermined peaks were seen at *m/z* 148 and *m/z* 273. These two undetermined peaks were also observed following injection of CSH without APAP. As shown in Fig. 9b on oxidation of APAP and mixing with CSH, two mass spectral peaks with low ion intensities were observed at *m/z* 184 and *m/z* 271. These *m/z* values correspond to the values indicative of the APAP-SC conjugate (Fig. 8). The selective ion scans of NAPQI, APAP-SC, and the two undetermined peaks *m/z* 148 and *m/z* 273 are shown in Fig. 10 at increasing oxidizing potentials, at 0.2 V increments. The NAPQI ion at *m/z* 108 behaved as described previously for the APAP-SG conjugate,

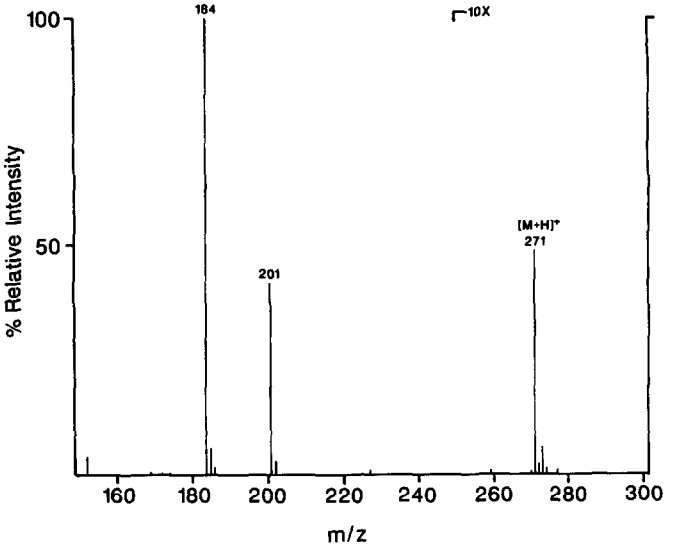

Fig. 8. Authentic sample of APAP-SC conjugate analysed by TSP MS in 0.1 *M* ammonium acetate.

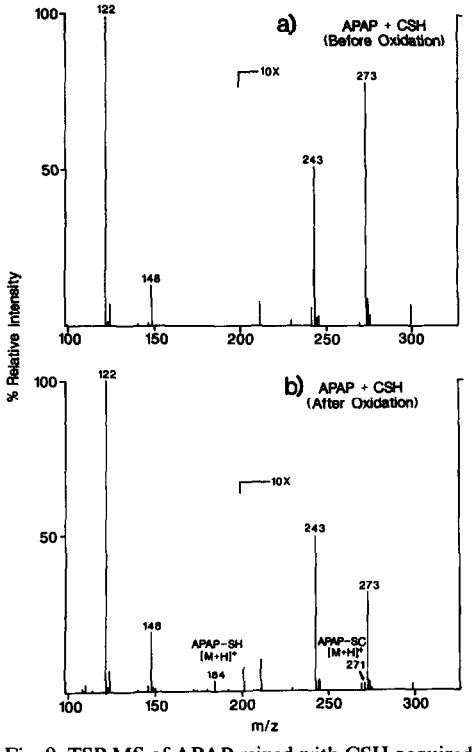

Fig. 9. TSP MS of APAP mixed with CSH acquired (a) before oxidation of APAP and (b) after oxidation of APAP. After oxidation of APAP, mass spectral peaks indicative of APAP-SC are observed for $[M+H]^+$ at m/z 271 and for the protonated thiol fragment ion at m/z 184.

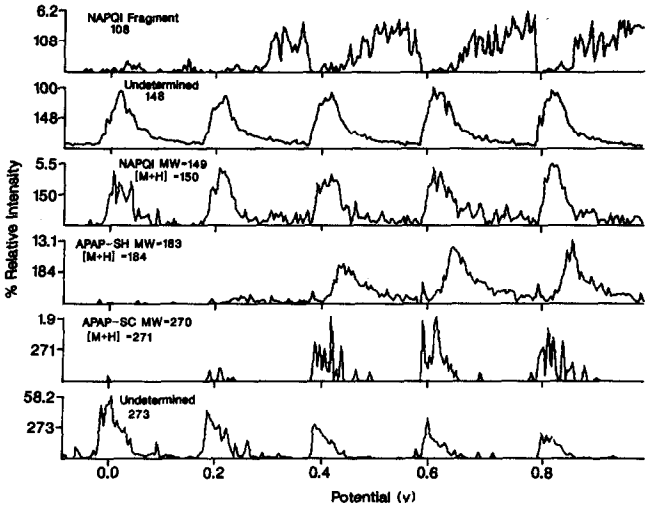

Fig. 10. Selective ion monitoring of ions indicative of NAPQI (*m/z* 150 and 108) and APAP-SC (*m/z* 271 and 184) shown at various-applied potentials. Ion intensities at *m/z* 148 and *m/z* 273 were due to undetermined ions.

but the *m/z* 150 ion intensity appeared even before APAP began to be oxidized. This ion intensity for *m/z* 150 prior to oxidation is possibly due to a sulfur isotope from the undetermined peak at *m/z* 148. This possibility is supported by the ratio of relative ion intensities (Fig. 10) for *m/z* 148 and *m/z* 150, which are 100% and 5.5%, respectively. The 100:5.5 ratio is approximately the ratio for the ^{32}S:^{34}S isotopes. The peaks corresponding to the ions associated with the APAP-SC conjugate appeared only after the APAP was electrochemically converted into NAPQI at +0.2 V. The $[M+H]^+$ ion at *m/z* 271 and the fragment at *m/z* 184 for APAP-SC appeared when 100 μl of the CSH solution was injected into the stream of NAPQI and mixed. The selective ion scan of one of the undetermined peaks in Fig. 10 demonstrated potential *vs.* ion intensity dependency; the *m/z* 273 species decreased in ion intensity at increasing oxidizing potential.

Glutathione conjugates have been succesfully observed as the $[M+H]^+$ ion by fast atom bombardment (FAB) MS for the APAP moiety[17], and FAB tandem mass spectrometry has also been reported[18]. A comparative study of TSP MS analyses of glutathione and cysteine conjugates has been carried out, and various other ionization modes in MS for these conjugates have been discussed[19]. In their comparative study, Parker *et al.*[19] noted that the ion intensities for $[M+H]^+$ ions were dependent on source conditions for these thermally labile conjugates. Optimizing the APAP-SG and APAP-SC ions for this study did require fine adjustment of the vaporizer and block temperatures, as well as the flow-rate into the mass spectrometer.

For the APAP-SG conjugate, ion intensities produced by SECYMS for the $[M+H]^+$ ion and the protonated APAP-SH fragment ion were similar to those previously reported[9]. The SECYMS of APAP-SC gave a much diminished ion intensity for the $[M+H]^+$ ion and protonated APAP-SH compared with the authentic sample. One difference for the APAP-SC conjugate *vs.* APAP-SG is that CSH was found to be

electrochemically oxidizable at a glassy carbon electrode. This oxidation occurred at a more anodic potential than that of APAP, so it did not have a direct effect on the formation of the APAP-SC conjugate. However, as pointed out in Fig. 10, one of the undetermined peaks (*m/z* 273) associated with CSH did give an indication that its ion intensity varied with electrode potential. It should be noted that the *m/z* 148 mass spectral peak also appeared in the APAP-SC authentic sample analysed by TSP MS (not shown in Fig. 8).

The electrochemistry of GSH and CSH has been studied by Thackrey and Riechel[20], who used glassy carbon electrode and mediators to enhance the electrochemistry. Cyclic voltammetry of GSH produced no discernible oxidative wave in 0.1 *M* KCl, which confirmed our observations of GSH in 0.1 *M* ammonium acetate, *i.e.* no oxidation detected by cyclic voltammetry. The reported voltammogram of CSH in 0.1 *M* KCl also indicated no oxidation[20]; however, in our experiments with differential pulse voltammetry we observed an oxidation wave at +1.45 V *vs.* Ag/AgCl in 0.1 *M* ammonium acetate without a mediator. The cyclic voltammogram of CSH gave only a rising background in 0.1 *M* ammonium acetate. Another factor that may explain the diminished ion intensities for APAP-SC by the SECYMS method compared with the authentic sample is that the rate of reaction for CSH conjugation with APAP may not be as rapid relative to GSH conjugation, which produced ion intensities similar to authentic samples reported in the literature[9].

The large ion intensities for CSH in Fig. 9, before and after oxidation of APAP, is due to the large excess of CSH used for the on-line electrochemical synthesis of APAP-SC. The mole ratio of CSH:APAP was *ca.* 20:1. The mole ratio of GSH:APAP to form the APAP-SG conjugate was *ca.* 10:1.

The study of APAP-SG and APAP-SC conjugation by SECYMS could be further enhanced by the utilization of an HPLC column between the electrochemical coulometric cell and thermospray mass spectrometer. This EC–HPLC–TSP MS experiment should be capable of separating unused reactants and multiple products, followed by characterization by TSP MS. For the example of APAP-SC, identification of *m/z* 148 and *m/z* 273 may be possible after separation by HPLC. Future studies will investigate the conjugates of *p*-aminophenol and related compounds and their electrochemical formation and detection by SECYMS.

CONCLUSION

The extension of SECYMS as a flow-injection reaction system has proved successful as a means to form on-line the GSH and CSH conjugates of APAP. The ability to generate conjugates in less than 1 s may prove invaluable in evaluating plausible conjugate formation prior to scaled-up synthesis for biochemical and animal studies. Furthermore, the electrochemical information retrieved, such as the number of electrons transferred, may enable investigators to confirm *in vivo* reaction mechanisms. An advantage for the mass spectrometrist utilizing TSMS is that a practically infinite amount of conjugates is available by this SECYMS method. This permits the optimization of the operating conditions for TSMS. These conditions may include varying the vaporizer and block temperatures along with flow-rates of liquid carriers. The SECYMS method may supply information on the chemical conditions for conjugate formation, as exemplified by the pH study of APAP-SG.

The SECYMS method is another technique that falls into the domain of spectroelectrochemistry. The coupling of mass spectrometry with electrochemistry allows the detection and characterization of reactive species, such as NAPQI, that may not be detected or characterized by any other MS method or, for that matter, any other spectroscopic technique. The simplicity and utility of SECYMS for observing redox reactions have been demonstrated by this study of on-line APAP conjugate formation.

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