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Evidence of the formation of direct covalent adducts of primaquine, 2-*tert*-butylprimaquine (NP-96) and monohydroxy metabolite of NP-96 with glutathione and *N*-acetylcysteine

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ABSTRACT

2-*tert*-Butylprimaquine (NP-96) is a novel quinoline anti-malarial compound with superior therapeutic profile than primaquine (PQ). Moreover, it is the first 8-aminoquinoline that is established to be devoid of methemoglobin toxicity. The purpose of the present study was to investigate covalent adduct formation tendency of PQ, NP-96 and their phase I metabolites with glutathione (GSH) and *N*-acetylcysteine (NAc). For the same, the two compounds were incubated in human and rat liver microsomes in the presence of trapping agents and NADPH. In a control set, NADPH was excluded, while a blank was also studied that was devoid of both NADPH and microsomes. The components in the reaction mixtures were initially separated on a C-18 column (250 mm × 4.6 mm, 5 μm) using a mobile phase composed of acetonitrile and 10 mM ammonium acetate in a gradient mode. The samples were then subjected to LC–MS^n and LC–HR-MS analyses, and data were collected in full scan MS, data dependent MS/MS, targeted MS/MS, neutral loss scan (NLS) and accurate mass (MS/TOF) modes. In a significant finding, both PQ and NP-96 themselves showed potential to bind covalently with GSH and NAc, as adducts were observed even in the control and blank incubations. Intense peaks corresponding to covalent adduct of mono-hydroxy metabolite of NP-96 with GSH and NAc were also detected in NADPH supplemented reaction solution.

1. Introduction

Most widely employed drugs for the treatment of malaria are chloroquine, primaquine (PQ) and artemisinin. However, all these are associated with one problem or the other. The use of chloroquine has been questioned owing to emergence of resistance and adverse effects [1]. PQ is employed for prophylaxis, but shows high incidence of toxicity, particularly in individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency [2]. Artemisinin is normally prescribed in combination therapy with other anti-malarials due to its shorter duration of action, but its use is also restricted due to the risk of development of bacterial resistance [3]. Thus considering the hurdles in therapy of malaria with established drugs, there is an urgent need for development of molecules with improved therapeutic activity and better safety to combat the dreadful onslaught of the parasite.

2-*tert*-Butylprimaquine (NP-96, Fig. 1) is a new experimental anti-malarial candidate, which is reported to be more potent and safer than PQ. It showed promising activity in vitro and in vivo, and more importantly it was devoid of methemoglobin (MetHB) toxicity [4]. Subsequent studies showed that it had schizonticidal action in vivo [5]. In continued investigations on this new medicinal entity (NME), the reactive metabolite formation potential was explored in the present study. The same was considered important keeping into view reports on the formation of direct covalent adducts of substituted quinolines with nucleophiles [6,7]. The study utilized various modes of LC–MS to detect and identify the adducts [8].

2. Materials and methods

2.1. Materials

NP-96 was synthesised and supplied by the Department of Medicinal Chemistry, NIPER, S.A.S Nagar, India. Rat liver microsomes (RLM) were purchased from BD Biosciences (San Jose, USA). Human liver microsomes (HLM) were supplied gratis by CellzDirect (Durham, USA). Nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH), di potassium hydrogen phosphate and potassium phosphate monobasic were purchased from Himedia Laboratories (Mumbai, India). Primaquine, glutathione (GSH) and *N*-acetylcysteine (NAc) and sodium formate were procured from Sigma–Aldrich Chemicals (Bangalore, India).

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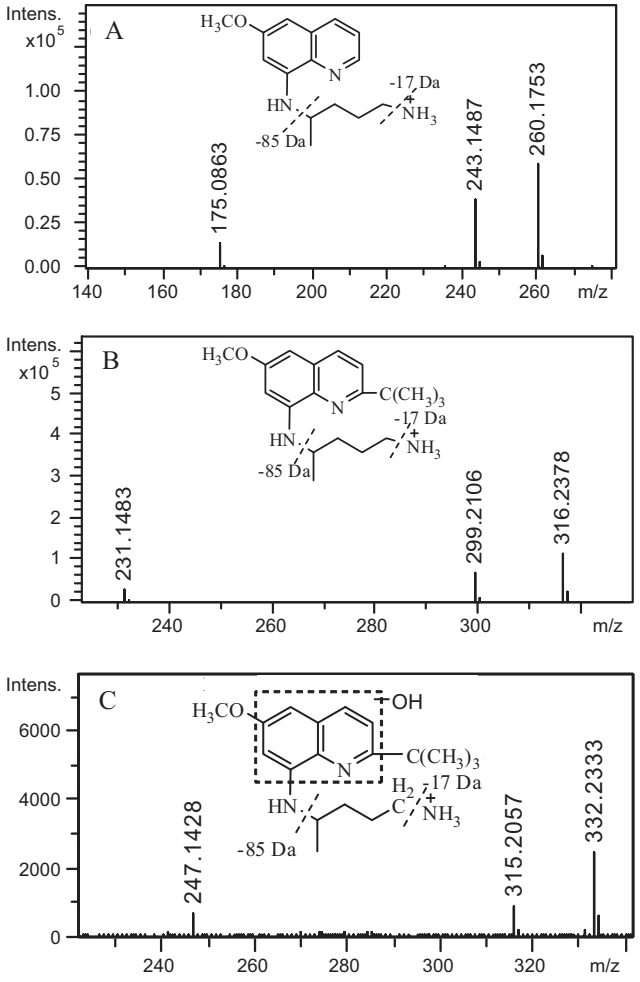


Fig. 1. HR-MS spectra, structures and sites of fragmentation of PQ (A), NP-96 (B) and monohydroxy-NP-96 (C).

Acetaminophen was obtained as a gratis sample from Arbro Pharmaceuticals (Delhi, India). HPLC grade acetonitrile (ACN) was procured from J.T. Baker (Phillipsburg, NJ, USA). Pure water was obtained from a water purification unit (Elga Ltd., Bucks, England). All other chemicals used were of analytical-reagent grade.

2.2. Incubation conditions and sample preparation

Liver microsomes (1 mg/ml), test compounds (10 μM), $MgCl_2$ (3.3 mM), NADPH (1.3 mM) and GSH or NAc (5 mM) were incubated at 37 ± 1 °C for 60 min. The control was a mixture devoid of NADPH, while, in blank both NADPH and microsomes were excluded. Also, acetaminophen was used as a positive control for all the microsomal incubations. After the presumed time, aliquots were withdrawn and subjected to sample preparation before analysis by LC–MS. Sample preparation step mainly involved protein precipitation using chilled ACN, followed by centrifugation at 9000 × *g* for 10 min (Eppendorf, Hamburg, Germany). The samples were further purified by solid phase extraction (SPE) using hydrophilic–lipophilic balance (HLB) cartridges (Waters, Milford, USA). The procedure involved cartridge conditioning with 1 ml methanol, followed by 2 ml water, loading of samples (1 ml), washing with 1 ml water and elution of the analytes with 0.5 ml ACN. Finally, aliquots from SPE were pooled and evaporated to dryness under a stream of nitrogen at 40 °C in a TurboVap (Caliper Life Sciences, Hopkinton, USA) and stored at −60 °C until analyses.

2.3. LC method development

During LC method development, the main consideration was good resolution between test molecules, their metabolites and adducts with GSH/NAc. To achieve the same, mobile phase was optimized in a manner that the test compound resolved at ~15 min on a C-18 column and other polar components eluted before it, with good resolution among themselves. For LC–MS studies, the methods were suitably modified by using a volatile buffer in the mobile phase. The desired separation was achieved on a Zorbax C-18 column (250 mm × 4.6 mm, 5 μm) from Agilent Technologies (Wilmington, DE, USA), employing a gradient mode and a mobile phase comprising of ACN and 10 mM ammonium acetate (pH 5.0). Details of the gradient methods are given in Table 1. In all the studies, the flow rate and column temperature were fixed as 0.35 ml/min and 25 °C, respectively.

2.4. LC–MS studies

LC–MS^n studies were performed on LTQ-XL™ linear ion-trap equipment (Thermo Electron Corporation, San Jose, USA) controlled with Xcalibur (version 2.0) software coupled to Accela™ HPLC

Table 1
HPLC gradient programmes and MS^n and MS/TOF parameters used for the test molecules (PQ and NP-96) and positive control (acetaminophen).

PQ		NP-96		Acetaminophen	
Time (min)	Buffer (%)	Time (min)	Buffer (%)	Time (min)	Buffer (%)
0 → 3	97	0 → 2	95	0 → 4	97
3 → 10	97 → 75	2 → 13	95 → 55	4 → 14	97 → 20
10 → 16	75 → 60	13 → 23	55 → 5	14 → 18	20
16 → 23	60 → 20	23 → 30	5	18 → 20	20 → 97
23 → 26	20 → 97	30 → 32	5 → 95	20 → 30	97
26 → 40	97	32 → 45	95		

MS^n parameters	PQ	NP-96
Sheath gas (arb)	30	30
Auxillary gas (arb)	10	12
Spray voltage (kV)	5	5
Capillary temperature (°C)	300	275
Capillary voltage (V)	48	31
Tube lens voltage (V)	40	65

MS/TOF parameters	PQ-GSH	PQ-NAc	NP-96-GSH	NP-96-NAc
Low mass (*m*/*z*)	100	80	100	80
Collision energy (eV/Z)	10	10	10	10
Collision RF (Vpp)	300	300	400	300
Transfer time (μs)	92	92	100	100

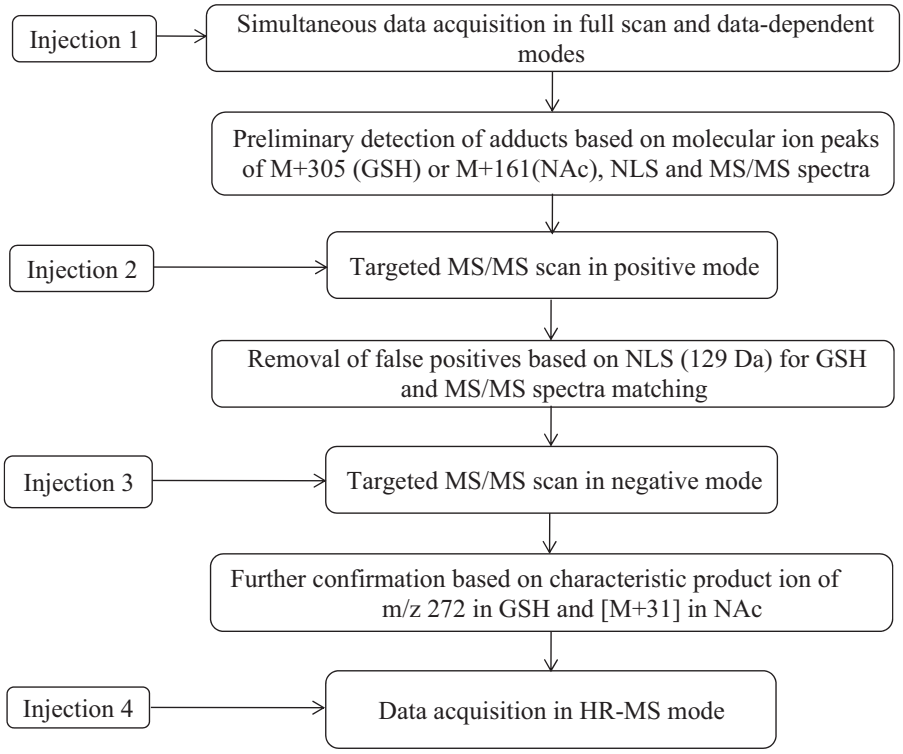


Fig. 2. Strategy employed for identification of GSH and NAc adducts. M = *m/z* value of parent compound.

system. Mass ionization parameters were optimized to get best possible fragmentation with acceptable sensitivity. Various detection modes, like selective reaction monitoring (SRM), neutral loss scan (NLS) and targeted MS/MS were utilised for the detection and identification of adducts. High resolution mass spectrometry (HR-MS) studies were carried out using a LC-MS/TOF instrument, which comprised of 1100 series HPLC system from Agilent Technologies (Waldbronn, Germany) and MicrOTOF-Q spectrometer from Bruker Daltonics (Bremen, Germany). The two were operated using combined software comprising of Hystar (version 3.1) and MicrOTOF Control (version 2.0). The calibration solution used for accurate mass studies was 5 mM sodium formate. All masses were corrected by internal reference ions having *m/z* values of 90.9766, 158.9641, 226.9515, 362.9263, 430.9138, 498.9012, 566.8886, 634.8760 and 702.8635. Both the above systems were used to study mass fragmentation behavior of the precursors, i.e., PQ, NP-96, GSH and NAc, and also for the analyses of experimental samples. The optimized mass parameters are given in Table 1.

2.5. Detection and identification of covalent adducts

For the detection and confirmation of GSH/NAc adduct(s), the protocol given in Fig. 2 was followed [9–14]. Each sample was subjected to data acquisition in full scan and data-dependent positive

Table 2
HR-MS data for PQ, NP-96, GSH and NAc.

Fragment	Experimental mass	Best possible molecular formula	Exact mass of most possible structure	Error in ppm
PQ				
$M+H^+$	260.1753	$C_{15}H_{22}N_3O^+$	260.1757	1.537
$(M+H^+)-NH_3$	243.1487	$C_{15}H_{19}N_2O^+$	243.1487	0.000
$(M+H^+)-C_5H_{11}N$	175.0863	$C_{10}H_{11}N_2O^+$	175.0866	1.713
NP-96				
$M+H^+$	316.2377	$C_{19}H_{30}N_3O^+$	316.2383	1.581
$(M+H^+)-NH_3$	299.2106	$C_{19}H_{27}N_2O^+$	299.2118	4.010
$(M+H^+)-C_5H_{11}N$	231.1483	$C_{14}H_{19}N_2O^+$	231.1492	3.893
Monohydroxy-NP-96				
$M+H^+$	332.2333	$C_{19}H_{31}N_3O_2^+$	332.2333	0.000
$(M+H^+)-NH_3$	315.2057	$C_{19}H_{28}N_2O_2^+$	315.2067	3.172
$(M+H^+)-C_5H_{11}N$	247.1428	$C_{14}H_{20}N_2O_2^+$	247.1441	5.260
GSH				
$M+H^+$	308.0892	$C_{10}H_{18}N_3O_6S^+$	308.0911	6.167
$(M+H^+)-H_2O$	290.0768	$C_{10}H_{16}N_3O_5S^+$	290.0805	12.755
$(M+H^+)-C_2H_5NO_2$	233.0580	$C_8H_{13}N_2O_4S^+$	233.0591	4.719
$(M+H^+)-C_5H_7NO_3$	179.0483	$C_5H_{11}N_2O_3S^+$	179.0485	1.117
NAc				
$(M+H^+)$	164.0358	$C_5H_{10}NO_3S^+$	164.0376	10.973
$(M+H^+)-H$	146.0258	$C_5H_8NO_2S^+$	146.0270	8.217
$(M+H^+)-C_2H_3O$	122.0278	$C_3H_8NO_2S^+$	122.0270	6.555

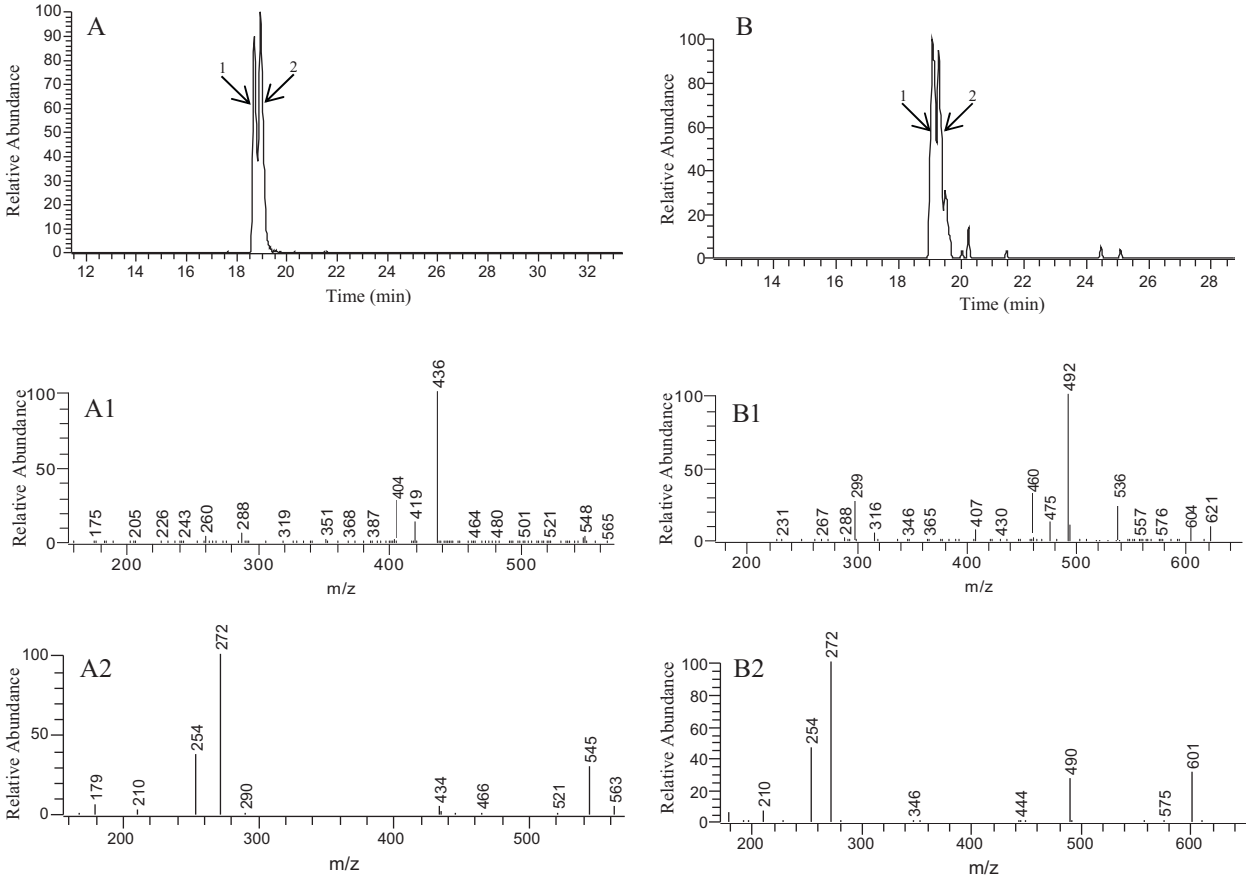


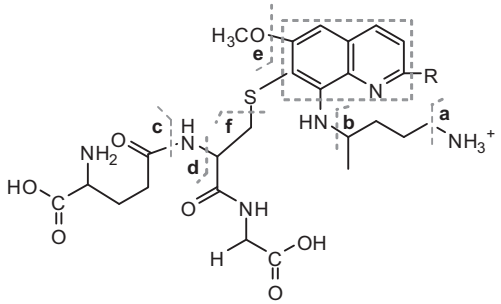
Fig. 3. Neutral loss (129 Da) chromatograms of PQ-GSH (*m*/*z* 565) (A) and NP-96-GSH (*m*/*z* 621) (B) along with representative MS/MS spectra in positive and negative ionization modes for PQ-GSH (*m*/*z* 565 and 563, respectively) (A1 and A2) and NP-96-GSH (*m*/*z* 621 and 619, respectively) (B1 and B2).

MS/MS, targeted MS/MS (both ESI positive and negative ionization modes), and HR-MS modes. Full scan and data-dependent MS/MS helped in predicting the number of possible adducts and their *m*/*z* values. The data acquired in positive ESI targeted MS/MS mode for the predicted values helped in identification of molecular ions of adducts with GSH and determining their number through NLS scan of 129 Da. The negative targeted MS/MS data reconfirmed the presence and number of adducts by the appearance of ions of *m*/*z* 272 and 254 for GSH adducts [15], and typical fragments in case of NAc adducts. Both studies thus assisted in eliminating false positives [16]. Finally, identification of each adduct was assisted by its HR-MS data, which were used to calculate their elemental compositions.

3. Results and discussion

3.1. Fragmentation patterns of test compounds and nucleophiles

The HR-MS spectra of PQ and NP-96 are shown in Fig. 1, along with their fragmentation profiles. Both compounds underwent facile two-step fragmentation in ESI positive mode, involving common losses of 17 Da (NH_3) and 85 Da (aliphatic side chain). In case



Adducts	R	M	M-a	M-b	M-c	M-d	M-(d+a)	M-(c+b)	M-(e+f)	M-(e+f+a)	M-(e+f+b)
PQ-GSH	H	565	548	480	436	419	404	351	260	243	175
NP-96-GSH	$C(CH_3)_3$	621	604	536	492	475	460	407	316	299	231

Key: M denotes $(M+H)^+$, a-f represent leaving fragments from adduct upon CID

Fig. 4. Structure of adducts of PQ and NP-96 with GSH along with superimposed fragmentation behavior.

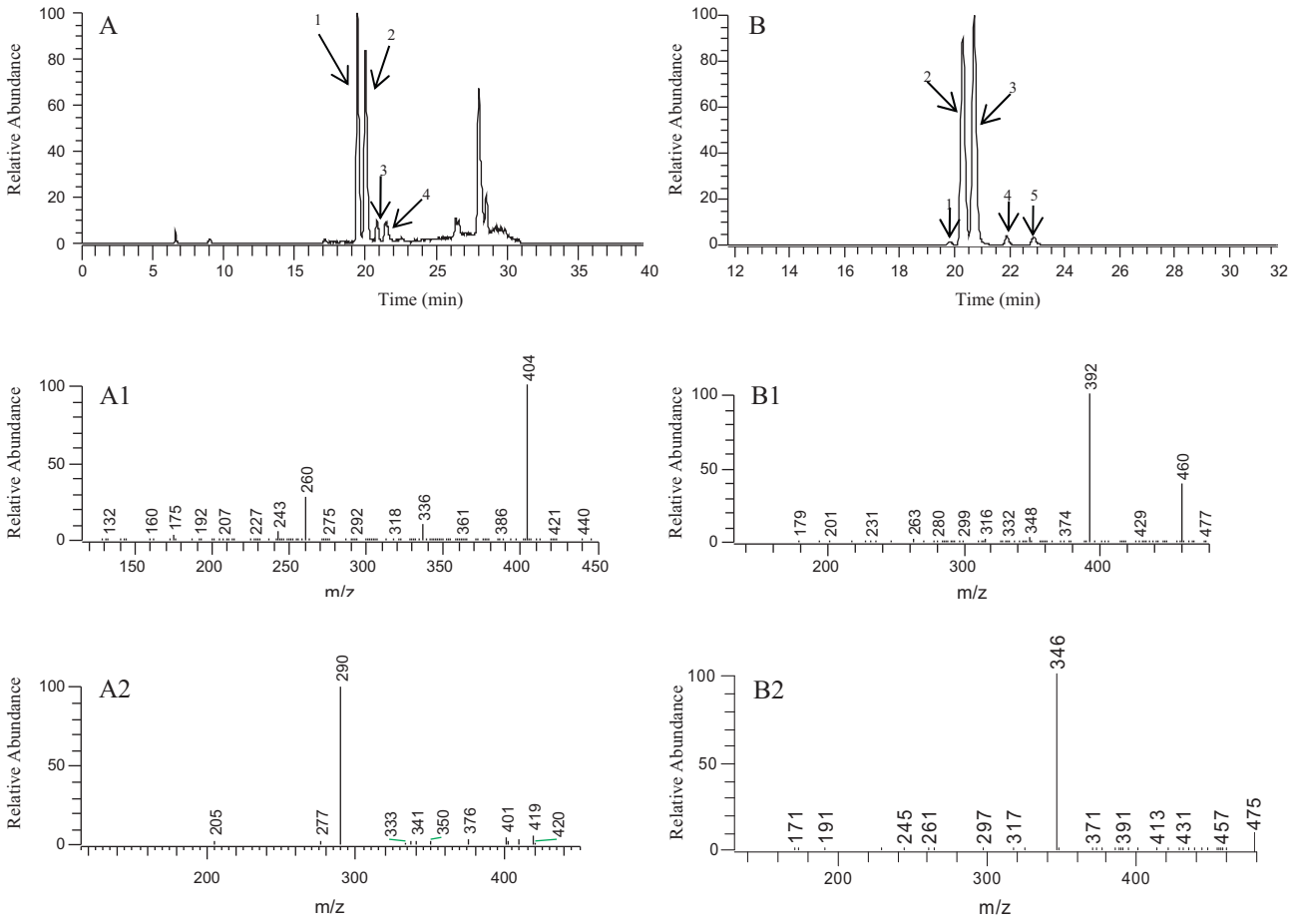


Fig. 5. TIC chromatograms of targeted MS/MS runs of PQ-NAc (*m/z* 421) (A) and NP-96-NAc (*m/z* 477) (B) along with representative MS/MS spectra in positive and negative ionization modes for PQ-NAc (*m/z* 421 and 419 respectively) (A1 and A2) and NP-96-NAc (*m/z* 477 and 475 respectively) (B1 and B2).

of GSH and NAc, the reported fragmentation data [17] were reproduced, wherein characteristic losses of 75 Da (glycine) and 129 Da (glutamic acid) were typical for GSH, while loss of acetyl moiety (42 Da) was unique for NAc. The HR-MS data for test compounds (PQ and NP-96) and nucleophiles (GSH and NAc) are given in Table 2.

3.2. Identification of covalent adducts of PQ, NAC and their metabolites

3.2.1. GSH adducts with PQ and NP-96

Before analyzing the in vitro samples containing test compounds, the viability of microsomal incubation was evaluated by using acetaminophen as a known substrate. The unique appearance of the GSH and NAc adducts (*m/z* 457 and 313, respectively) confirmed the enzymatic conversion of acetaminophen to iminoquinone following by its trapping by the nucleophiles [18]. Afterwards, the reaction samples of PQ and NP-96 with GSH were subjected to data dependent mass acquisition to check for the formation of adducts. Mass peaks of *m/z* 565 and 621 gave strong indication of the presence of adducts of GSH directly with PQ and NP-96, respectively. Subsequently, NLS scans were acquired upon targeted MS/MS analyses to look for the loss of 129 Da, typical of GSH adducts [17]. In case of both PQ and NP-96, two isomeric peaks of GSH adducts were observed, as shown in Fig. 3A and B, respectively. These were observed in both reaction and control incubations, which showed that both PQ and NP-96 directly formed adducts with GSH, even without undergoing metabolism. To verify the same, respective MS/MS spectra were recorded in positive and negative ionization modes. As shown in Fig. 3A1, in positive ionization mode, the expected fragment of *m/z* 436 for PQ-GSH adduct was observed on the loss of 129 Da of glutamic acid. The same 129 Da loss was also observed for NP-96-GSH adduct, with most abundant fragment appearing in this case at *m/z* 492 (Fig. 3B1). In both the cases, fragments pertaining to PQ and NP-96, i.e., *m/z* 243 + 305, 175 + 305 (Figs. 3A1 and 4), and *m/z* 299 + 305, *m/z* 231 + 305 (Figs. 3B1 and 4), respectively, were also observed. Another confirmation came through MS/MS spectra in negative ionization mode (Fig. 3A2 and B2 for PQ and NP-96, respectively), where characteristic fragments of *m/z* 272 and 254, typical of GSH adducts, were observed [15]. The above was supported by HR-MS data (Table 3). Further confirmation was provided by justification of the mass fragmentation pattern for adducts, as shown in Fig. 4.

3.2.2. NAc adducts with PQ and NP-96

Similar to the GSH adducts, covalent binding of PQ and NP-96 with NAc were identified in the nucleophile supplemented incubations. Initially, the mass values of the NAc adducts were predicted based on the already collected GSH adduct data in the positive ionization mode. The values were *m/z* 421 and 477 for PQ and NP-96, respectively. As neutral loss of 129 Da (typical for GSH adducts) could not happen in NAc in the positive mode, targeted MS/MS analyses for ions of *m/z* 421($M+H^+$)/419($M-H^-$) and *m/z* 477($M+H^+$)/475($M-H^-$) were performed for PQ and NP-96, respectively, in both positive and negative modes. The resultant total ion chromatograms (TIC) and representative MS/MS spectra are given in Fig. 5. As evident, multiple peaks were observed for the predicted masses in both the reaction and control samples for PQ and NP-96. They produced similar fragmentation pattern, indicating that

Table 3
HR-MS data for PQ-GSH, NP-96-GSH, PQ-NAc and NP-96-NAc adducts.

Fragment	Experimental mass	Best possible molecular formula	Exact mass of most possible structure	Error in ppm
PQ-GSH				
$M+H^+$	565.2413	$C_{25}H_{37}N_6O_7S^+$	565.2439	4.599
$(M+H^+)-NH_3$	548.2171	$C_{25}H_{34}N_5O_7S^+$	548.2173	0.364
$(M+H^+)-C_5H_7NO_3$	436.2027	$C_{20}H_{30}N_5O_4S^+$	436.2013	3.209
NP-96-GSH				
$M+H^+$	621.3036	$C_{29}H_{45}N_6O_7S^+$	621.3065	4.667
$(M+H^+)-C_5H_7NO_3$	492.2681	$C_{24}H_{38}N_5O_4S^+$	492.2639	8.532
PQ-NAc				
$M+H^+$	421.1885	$C_{20}H_{29}N_4O_4S^+$	421.1904	4.511
$(M+H^+)-NH_3$	404.1620	$C_{20}H_{26}N_3O_4S^+$	404.1639	4.701
$(M+H^+)-C_5H_{11}N$	336.0998	$C_{15}H_{18}N_3O_4S^+$	336.1013	4.463
NP-96-NAc				
$M+H^+$	477.2529	$C_{24}H_{37}N_4O_4S^+$	477.2530	0.209
$(M+H^+)-NH_3$	460.2252	$C_{24}H_{34}N_3O_4S^+$	460.2265	2.824
$(M+H^+)-C_5H_{11}N$	392.1717	$C_{19}H_{26}N_3O_4S^+$	392.1639	19.889

these were isomeric, similar to the observation in GSH. The MS/MS spectra in positive ESI mode (Fig. 5A1 and B1) for both PQ and NP-96-NAc adducts showed the presence of characteristic losses of 17 Da and 85 Da. The same outcome was obtained from targeted MS/MS analyses in negative ESI mode, which showed characteristic fragment ions of *m/z* 290 and *m/z* 346 in case of PQ and NP-96 (Fig. 5A2 and B2), respectively. These fragments were formed by the neutral loss of 2-acetamidoacrylic acid moiety ($C_5H_7NO_3$). Further, HR-MS studies also confirmed the same (Table 3). Fig. 7 shows the fragmentation of probable adducts, based on collision induced dissociation (CID) data.

3.2.3. GSH and NAc adducts of phase I metabolites of the test compounds

The TIC and MS/MS spectra of microsomal incubation of NP-96 (Fig. 6) supplemented with NAc showed two isomeric peaks of *m/z* 493. As the same were absent in the blank and control incubations, it indicated covalent adduct formation between the metabolite and NAc. However, the corresponding GSH adducts were not seen perhaps due to their low intensity in MS. The formation of metabolite-NAc adducts was supported by their fragmentation pattern, which was very similar to NP-96-NAc adducts, with anticipated difference of 16 Da for all the fragments. Also, a typical fragment of *m/z* 362 (Fig. 6A2) was seen in the negative ionization mode, similar in line with *m/z* 346 in case of NP-96-NAc adduct (Fig. 5B2). The probable structure of adduct and its fragmentation pattern are given in Fig. 7. The corresponding adduct was not seen in GSH supplemented incubation. It was perhaps due to the lower analytical sensitivity of GSH adduct of the metabolite as the intensity of GSH adduct of the test compounds was also significantly less in LC–MS, as compared to their NAc adduct.

Surprisingly, in the case of microsomal incubations of PQ, we could not detect any reactive metabolites in the sample. Although, in vitro metabolism of PQ is reported elsewhere [19], they have also not mentioned formation of hydroxyl metabolites corresponding to the reactive metabolite of NP-96. Moreover, we could not observe the known carboxy metabolite of PQ, may be due to the lesser substrate/protein concentration used in our experiments. Further, to minimize false negatives in this case, efficiency of SPE extraction was validated by comparing the profile from both SPE sample and direct protein precipitation sample. In both cases, the metabolic profiles were found to be similar.

3.2.4. Relevance of the study with respect to the toxicity potential of PQ and NP-96

In literature, direct covalent binding with nucleophiles (GSH and NAc) has been reported for 4-sulfonyl-2-pyridone ring systems [20] and more specifically with methoxy 4-O-aryl quinolines [7], similar to our observations in this study with 8-amino substituted quinolines. This suggests that there may be some role of electrophilicity of PQ with respect to toxicity shown by it. This even means a similar toxicity potential for NP-96. However, conclusive assessment would require relative and quantitative investigation of adduct formation potential between the two compounds. These findings may pave the way for further modifications in PQ and NP-96 structures, considering that inherently electrophilic compounds are likely to cause toxicity because of their ability to alkylate proteins, DNA and/or GSH. However, the chemical modification should not affect pharmacological efficacy of these compounds.

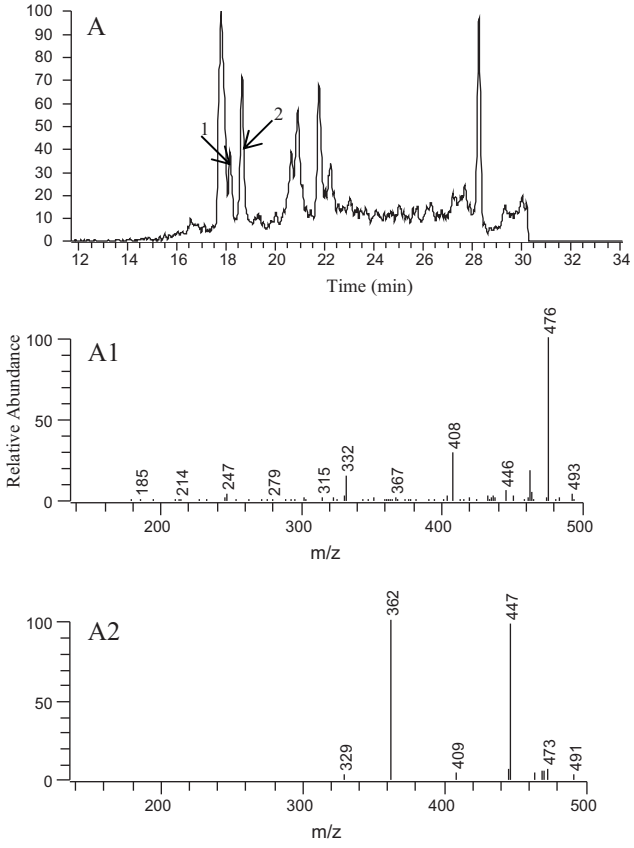
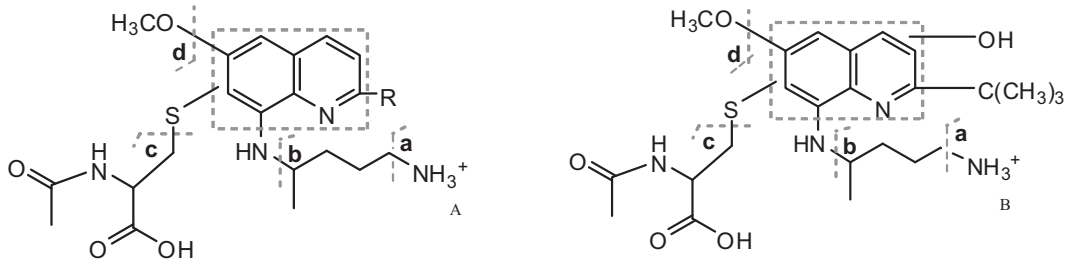


Fig. 6. TIC chromatograms of monohydroxy-NP-96-NAc (*m/z* 493) along with representative MS/MS spectra in positive (*m/z* 493) (A1) and negative (*m/z* 491) (A2) ionization modes.



Adducts	R	M	M-a	M-b	M-c	M-(c+a)	M-(d+c)	M-(d+c+a)	M-(c+b)	M-(d+c+b)
PQ-NAc	H	421	404	336	292	275	260	243	207	175
NP-96-NAc	$C(CH_3)_3$	477	460	392	348	331	316	299	263	231
Monohydroxy-NP-96-NAc	-	493	476	408	364	-	332	315	-	247

Key: M denotes $(M+H)^+$, a-d represents leaving fragments from adduct under CID

Fig. 7. Structure of adducts of PQ-NAc and NP-96-NAc (A) and monohydroxy-NP-96-NAc (B) with superimposed fragmentation behavior.

4. Conclusions

The following were the significant outcomes of the study:

(1) Direct covalent binding of PQ and NP-96 was observed with GSH and NAc. The adducts were formed in all the in vitro incubates, irrespective of the presence of microsomes and NADPH, though adducts were intense in microsomal incubates. This direct binding nature of 8-aminoquinoline compounds with nucleophiles, which is being reported for the first time to our knowledge, may be a possible reason for the toxicity behavior of PQ [21].
(2) NP-96 showed liability to be converted to a mono-hydroxy metabolite, which was also observed to form adducts with NAc, similar to its parent.
(3) NAc behaved as a better trapping agent for PQ and NP-96 than GSH perhaps due to its enhanced sensitivity in MS system, though it did not confirm relative tendency of the nucleophiles to interact with the compounds involved. Hence, there is a requirement of the use of established absolute quantitative methods [22] to determine differential adduct formation behavior of PQ and NP-96 with the nucleophiles.

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