THE IN VIVO FORMATION OF AMINE ADDUCTS OF THE PHENOLIC ANTIOXIDANT BHT FROM DIETARY SOURCES

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Introduction

2,6-Di-t-butyl-4-methylphenol or butylated hydroxytoluene (BHT) is a widely used phenolic antioxidant in food and other consumer products. Some phenolic antioxidants, including BHT, are known to exert physiologically significant effects in a number of species and their presence in water has been implicated in changes to fish gender distributions and reproduction. While some of the activity of these compounds may be attributed to biomimicry of estrogenic substances, the ability for para-methyl substituted phenolics to form adducts via a quinone methide intermediate is also known.

During routine drug testing of racing horses, three unknown compounds were detected in a large number of urine samples by GC-NPD and GCMS following solvent extraction. The compounds occurred together but their relative abundance varied from sample to sample in which they were detected. In each sample, BHT and BHT-quinone methide (BHT-QM) were also found. No other common link connected any of the samples in which the unknowns were observed.

Discussion and Conclusion

The basic extraction and gas chromatographic analysis of horse urine, collected for routine drug testing, showed the presence of three previously unknown compounds (Fig 1). The compounds BHT and BHT-QM were also detected in each of the samples containing any or all of the irregularities. BHT is widespread as an anti-oxidant used for the protection of equestrian food and other products and so its detection in urine was unremarkable.

The EI mass spectra of the unknown compounds are summarised in Table 1. PICI GCMS confirmed that the molecular ions are at m/z 263, 289 and 303 respectively and methyl iodide derivatisation confirmed the presence of one active hydrogen. The data allowed the tentative identification of the compounds as amine adducts of BHT and the structures were subsequently confirmed by independent synthesis.

The anti-oxidant effect of substituted phenols, such as BHT, is proposed to proceed via the formation of an endoperoxide or 4-hydroperoxy-2,5-dienone intermediate. The quinone methide is a powerful alkylating agent capable of reacting with suitable nucleophiles by a Michael type addition. BHT-QM has been shown to form protein adducts by reacting with pendant amine groups.

By extension of this observed reactivity, the amine adducts identified here might be the products of the addition of BHT-QM to simple amines. These adducts combine a sterically hindered aromatic ring attached to an alkylamino moiety. The formation of the compounds accounts for only a small percentage of the total BHT excreted and so their physiological activity may be unrecognized in vivo or be attributed to the more abundant BHT or other co-administered substances.

Consideration of the ability of phenolic antioxidants to form small molecule adducts that possess modified functional chemistry in aqueous environments may provide new avenues for describing the physiological activity of the parent antioxidant.

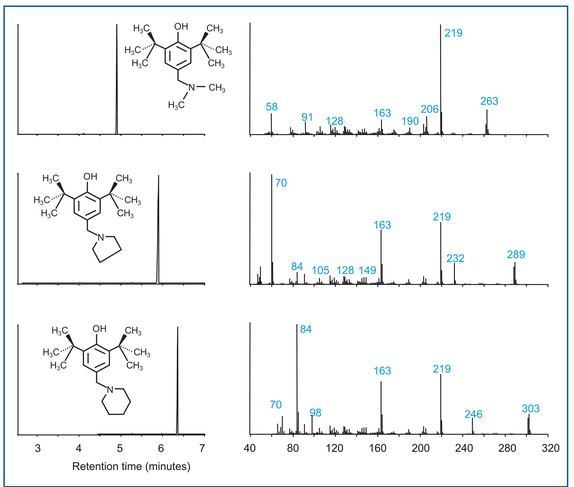


Figure 1: GC and MS data for three BHT artefacts isolated from equine urine. Compounds were identified as N-(3,5-di-t-butyl-4-hydroxyphenyl)-N,N-dimethylamine (top), N-(3,5-di-t-butyl-4-hydroxyphenyl)methylpyrrolidine (middle) and N-(3,5-di-t-butyl-4-hydroxyphenyl) methylpiperidine (bottom).

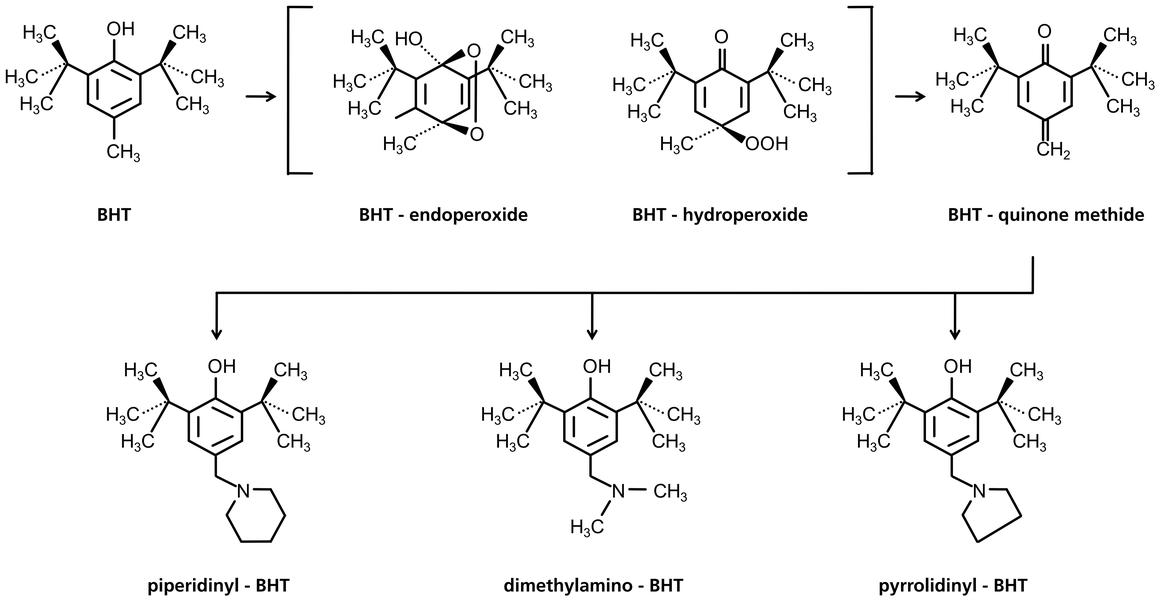
Compound	M	fragment ions (% abundance)							
BHT-QM	218	161	203	108	91	175	218	163	189
	100	*47*	*40*	*36*	*32*	*31*	*27*	*20*	
pyrrolidinyl-BHT	303	84	163	219	98	203	246	303	302
	100	*51*	*48*	*15*	*11*	*10*	*10*	*9*	
O-methyl derivative	317	177	84	233	317	178	234	316	260
	100	*91*	*82*	*41*	*32*	*26*	*20*	*16*	
piperidinyl-BHT	289	70	163	219	288	203	289	232	84
	100	*48*	*47*	*13*	*13*	*12*	*10*	*10*	
O-methyl derivative	303	177	233	70	178	234	303	219	203
	100	*41*	*33*	*20*	*15*	*11*	*10*	*9*	
N,N -dimethylamino-BHT	263	219	263	220	203	163	58	262	205
	100	*27*	*24*	*22*	*20*	*20*	*14*	*10*	
O-methyl derivative	277	177	233	178	234	276	277	262	219
	100	*50*	*23*	*21*	*7*	*6*	*2*	*2*	

Table 1 : Eight peak index of EI mass spectral data for artefacts formed from BHT.

EXPERIMENTAL CONDITIONS

- *Urine samples (5 mL), or blank urine samples spiked with 100 ng/mL of independently synthesized N-(3,5-di-t-butyl-4-hydroxyphenyl)methylpyrrolidine or N-(3,5-di-t-butyl-4-hydroxyphenyl)-N,N-dimethylamine, were diluted with potassium phosphate buffer (2.5 mL, 0.1 M, pH 6.3) and the pH adjusted to 6 with the addition of 2.0 M sodium hydroxide solution or 1.0 M aqueous hydrochloric acid, as required. The diluted urine was enzyme hydrolyzed with beta-glucuronidase (500 IU, E. coli, Sigma, St Louis, USA) for 2 hours at 50 °C. The hydrolyzed urine was adjusted to pH 9.5 with the addition of 2.0 M sodium hydroxide and centrifuged for 5 minutes at 3000 rpm to remove suspended solids. The clear solution was transferred to a screw-capped culture tube and extracted with 1-chlorobutane (5 mL) on a rotary mixer at 60 rpm for 5 min. The organic phase was separated off, dried over anhydrous sodium sulphate and evaporated to dryness under a stream of nitrogen at 20 °C. The dried residues were taken up in ethyl acetate (100 µL) and analyzed by GC-NPD and EI GCMS. Extracts were optionally treated with potassium carbonate (2 mg), methyl iodide (50 µL) and acetone (50 µL) at 60 °C in a sealed tube for 30 minutes then cooled, diluted to 2 mL with saturated sodium tetraborate buffer and extracted with dichloromethane (2 mL). The organic phase was separated off, dried over anhydrous sodium sulphate and evaporated to dryness under a stream of nitrogen at 20 °C prior to reconstitution for GC or GCMS analysis as described above.*
- *Electron impact (EI) GCMS was performed on a Hewlett Packard 5890 GC - 5971 MSD (Palo Alto, USA). The GC was equipped with a BPX5 column (SGE, Melbourne Australia, 12 m x 0.2 mm x 0.25 µm film thickness) with an inlet pressure of 5 psi.*
  - *The oven temperature was held at 75 °C for 2 minutes then heated at 30 °C/min to 300 EC with a final holding time of 9.0 minutes.*
  - *Injection was splitless.*
  - *The detector temperature was 280 °C and the injector temperature 250 °C.*
  - *A scan range of 50 to 500 Da at 1.2 scan/sec was used.*
- *Gas chromatography with Nitrogen-Phosphorous Detection (GC-NPD) was performed on a Hewlett Packard 5890 GC equipped with a NP detector, HP7673A Auto-injector, HP7673A controller, and HP 3396A Integrator. Chromatographic conditions were identical to those described above for GCMS.*
  - *The detector temperature was maintained at 300 °C.*
  - *Gas flow rates to the detector were air (120 mL/min), hydrogen 4.5 mL/min and make-up gas (nitrogen, 30 mL/min).*
  - *The NPD bead current was adjusted to a baseline output of 25 picoamps.*

PROPOSED FORMATION OF AMINE ARTEFACTS FROM BHT



Standards of N-(3,5-di-t-butyl-4-hydroxyphenyl)methylpyrrolidine or N-(3,5-di-t-butyl-4-hydroxyphenyl)-N,N-dimethylamine were synthesized by the author. Synthesis details and confirmatory data are available on request. Extraction and analysis performed by the author while appointed as Principal Scientist at Racing Analytical Services Limited, Flemington, Australia.

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