Comparative Study of Hops-Containing Products on Human Cytochrome P450-Mediated Metabolism

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ABSTRACT: The potential for 15 different ales (6), ciders (2 apple and 1 pear), and porters (6) and 2 non-alcoholic products to affect cytochrome P450 (CYP)-mediated biotransformation and P-glycoprotein-mediated efflux of rhodamine was examined. As in our previous study, a wide range of recovered nonvolatile suspended solids dry weights were noted. Aliquots were also found to have varying effects on biotransformation and efflux. Distinct differences in product ability to affect the safety and efficacy of therapeutic products confirmed our initial findings that some porters (stouts) have a potential to affect the safety and efficacy of health products metabolized by CYP2D6 and CYP3A4 isozymes. Most products, except 2 of the ciders and the 2 non-alcoholic products, also have the potential to affect the safety of CYP2C9 metabolized medications and supplements. Further studies are required to determine the clinical significance of these findings.

KEYWORDS: beer, hops, cytochrome P450 3A4, P-glycoprotein

INTRODUCTION

Adverse events associated with food, natural health products (NHPs), or drug–drug interactions are a major concern to regulatory bodies, health care professionals, and patients.[1–6] For example, some NHPs, such as St. John's wort, ginseng, grapefruit juice, danshen, Echinacea, and licorice, interact with other health products, and some have direct effects on the cardiovascular or hemostatic system.[7] Among the main issues are the lack of scientific evidence for the safety and efficacy of these products when taken with other health products and the under-reporting of adverse drug reactions.[7] The overall impact of these interactions is underestimated and may have a profound impact on health care resources and quality of life for the consumer.[1,3,4,7]

In our preliminary study, 35 national and international brands of ales and lagers, including a number of specialty bitter (porter and stout), coffee, ice, wheat, Pilsner, and hemp seed products, were examined for their potential to affect human cytochrome P450 (CYP)-mediated metabolism.[8] Wide variance was detected in contents of alcohol, nonvolatile suspended solids (NVSS), and hop acids and in the potential to affect CYP-mediated metabolism and Pgp-mediated efflux transport. Many of the examined products affected CYP2C9-mediated metabolism, and the two stouts examined markedly affected CYP3A4-mediated metabolism, suggesting that some products may have the capacity to affect drug safety. One of the early drivers for this study was recognition of the Mediterranean diet rich in foodstuffs, such as vegetables, herbs, legumes, and fruits. These have high secondary metabolite content and antioxidant levels and when combined with moderate alcohol consumption contribute to reduced cardiovascular risk.[9,10] Although there has been an increase in the number and range of adverse events with foods or medicinal plants being reported to health authorities and published in the scientific literature, the extent to which interaction with alcoholic beverages could have been a contributing factor is not known. The findings from the preliminary study[8] were consistent with those reported earlier that hop constituents are potent and selective inhibitors of CYP.[11–15]

Hop acids are a family of bitter compounds derived from the hop plant (Humulus lupulus) and have been reported to exert a wide range of in vitro and in vivo effects, including increasing the expression of CYP detoxification enzymes.[14,16–19]

The present study was undertaken to extend the findings of the preliminary study by examining a wider range of ale types, which include bitters, pale ale, and porter (including stout) representative of Canadian and international markets (Table 1) and included ciders and non-alcoholic lagers. The ciders were included as non-hops-containing controls, and the <0.5% alcohol lagers were included as non-alcohol controls. The chemical characterization of six common bitter humulones of beer hops in these products is being reported elsewhere.[20]

MATERIALS AND METHODS

Experimental Products. All products were obtained locally from commercial outlets. Each product was catalogued with a Nutraceutical Research Programme (NRP) number and an intact sample archived.

NVSS. Dry weight was determined by taking three 10 mL aliquots of each product and reducing them to a constant weight in a drying oven

Table 1. Country Source and Alcohol Content Information Taken Directly from the Product Label of the Hops Products Studied[a]

NRP number	product	source country	alcohol percentage (%)	type	dry weight (mg/mL)
367	Alexander Keiths Red Amber	Canada	5.0	ale	48.5 ± 1.30
368	Greene King Abbot	England	5.0	ale	55.7 ± 3.04
369	Hockley Dark	Canada	5.0	ale	84.3 ± 6.44
370	Riggwelter	England	5.7	ale	50.2 ± 3.82
371	Trafalgar Abbey Belgian Spiced Ale	Canada	6.2	ale	79.3 ± 2.57
372	Wellington Best Bitter	Canada	4.0	ale	36.4 ± 1.20
373	Magners Irish Cider (apple)	Ireland	4.5	cider	50.7 ± 1.53
374	William's Sir Perry Pear Cider	England	6.0	cider	77.4 ± 1.71
375	Strongbow Apple Cider	England	5.3	cider	49.8 ± 1.23
376	Dragon	Jamaica	7.5	stout	83.5 ± 4.10
377	Fuller's London Porter	England	5.4	porter	75 ± 3.32
378	John By Imperial	Canada	6.7	stout	64.6 ± 3.35
379	Sinha	Sri Lanka	8.0	stout	63.7 ± 2.58
380	St. Ambriose Oatmeal	Canada	5.0	stout	87.2 ± 2.95
381	Trafalgar Smoked Oatmeal	Canada	5.0	stout	77 ± 3.00
382	Exel	Canada	<0.5	lager	44.9 ± 5.95
383	Red Brew	Canada	<0.5	lager	52.2 ± 5.55

[a] The ciders were included as non-hops-containing controls, and the <0.5% alcohol lagers were included as non-alcohol controls. The dry weight of non-volatile compounds was determined using a 10 mL aliquot, in triplicate, reduced to a constant weight at 37 °C [mean ± standard deviation (SD)]. Stouts and porters are dark beers; the term stout was historically applied to strong (higher alcohol content) products but now is occasionally used as a synonym for dark beer.

overnight. The weight boats were reweighed, and the mean difference was determined for each product.

Human CYP-Mediated Metabolism. Aliquots (10 μL) of stock solutions of the undiluted products were screened for their ability to inhibit CYP3A4, CYP2D6, and CYP2C9 (BDGentest) metabolism of the non-fluorescent dibenzylfluorescein (DBF) or 7-ethoxy-3-cyanocoumarin (CEC) and 7-methoxy-4-trifluoromethyl-coumarin (MFC) substrates to the fluorescent metabolite using an *in vitro* fluorometric microtiter plate assay (CytoFluor Series 4000 multi-well plate reader). Briefly, assays were performed with 10 μL of product in clear-bottom, opaque-welled microtiter plates (96 well, Corning Costar).[8] Control and control blank wells contained 5% ethanol, and test and test blank wells contained the product. All wells tested contained distilled water, β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH, Sigma Chemicals), and the enzyme substrate DBF, CEC, or MFC (final concentration of 1 μM/well), for a total reaction volume of 200 μL. Control and test wells also contained live isozyme, within human microsomes, in phosphate buffer solution (PBS; 0.5 M, pH 7.4), and control blank and test blank wells contained denatured isoenzyme in PBS. Comparative testing of CYPs was previously performed with balanced amounts of specific activity and protein content using a BDGentest control.[21] Fluorescence was measured at 485 nm excitation and 530 nm emission, with a gain of 50. Because of the importance of CYP3A4-mediated metabolism, testosterone was tested as a secondary substrate as above but with an addition of 3.3 mM MgCl$_2$. Reactions were stopped with the same volume of ice-cold acetonitrile and filtered through polytetrafluoroethylene (PTFE) filters (0.45 μm pore; Chromatographic Specialties, Inc., Brockville, Ontario, Canada). An Agilent 1100 Series high-performance liquid chromatography (HPLC) system with a Supelco LC-BD C18 column (5 μm practical size, 250 × 4.6 mm; Supelco, Ottawa, Ontario, Canada) was used to monitor the reaction, and a diode array detector was set at 245 nm. For all assays, microsomes were rapidly thawed and mixed gently with the substrate solution. All microsomes were stored at −80 °C until used and were not subjected to more than two freeze–thaw cycles. All samples were prepared in triplicate and repeated at least once under reduced lighting conditions to reduce any risk of either photoactivation or photodecomposition of botanical

constituents or co-factors (NADPH), with the resultant percent inhibition calculations based on the mathematical combinations for the differences in fluorescence between the test/test blank wells and the mean difference between each control and blank well. A positive control using the CYP inhibitor ketoconazole (and bifonazole, sulfaphenazole, and tranylcypromine) at a final concentration of 0.5 μg/mL was run with every assay.

Human P-Glycoprotein-Mediated Efflux Transport. Human peripheral blood monocytes from patients with acute monocytic leukemia (ATCC TIB-202) were routinely subcultured, grown, and maintained horizontally in 80 cm^2 cell culture flasks (Nunclon) in a Fisher Scientific 605 incubator set to 37 °C and 5% CO$_2$ in RPMI 1640 supplemented with 10% fetal bovine serum, 1% sodium pyruvate, 1% penicillin–streptomycin (the above supplied by Invitrogen), and 1% β-mercaptoethanol (Sigma). Cell culture health was routinely assessed by direct observation of cultured cells via microscopy (Leica DMIL) for contamination and cell density count comparisons. Suspended cells in culture medium were centrifuged (Hettich Zentrifugen, Rotofix 32) at 300 RCF at room temperature for 10 min and then resuspended in the fresh culture medium at the concentration of 300 000 cells/978 μL for experiments.

Briefly, the Rh123 assay was carried out in the following manner with 978 μL of THP-1 cells plated into a 24-well cell culture plate (Falcon), along with 12 μL of Rh123 (final concentration of 150 ng/mL) and 10 μL of the sample production. After an incubation time of 18 h, cells were collected in 1.5 mL microfuge tubes, washed twice with PBS (0.5 M, pH 7.4), and centrifuged at 500 RCF (Eppendorf Centrifuge 5415R) for 5 min each. After the final wash and centrifuge at 500 RCF, the supernatant was discarded and the cell pellet was resuspended in 1 mL of fresh PBS. A 300 μL aliquot of the cell suspension, in triplicate, was measured on a 96-well plate at 37 °C for reading, using a fluorometric microtiter plate assay (CytoFluor Series 4000 multi-well plate reader). Fluorescence was measured at 485 nm excitation and 530 nm emission, with a gain of 60. All samples were prepared in triplicate and repeated at least once under reduced lighting conditions.

High-Performance Liquid Chromatography–Atmospheric Pressure Chemical Ionization–Mass Spectrometry Detector (HPLC–APCI–MSD). A 10 mL aliquot of each beer sample was

5160

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centrifuged at 1000g for 15 min at room temperature. A total of 1 mL of each sample was filtered through a PTFE membrane to a HPLC vial, and 90 μL of each beer sample and 0.9 mL of hops standardized extract were injected into HPLC–MS through an autosampler. An Agilent 1100 series liquid chromatography (LC)–MSD system (Agilent Technologies, Inc., Santa Clara, CA), equipped with an online degasser, a quaternary pump, an autosampler, a column thermostat, and an APCI–MSD technique, was used for the analyses. The separations were performed on a reversed-phase Synergi Fusion C18 column, 150 × 3.0 mm inner diameter and 4 μm particle size (Phenomenex, Inc., Torrance, CA). The flow rate of solvents (A, acetonitrile; B, 4% aqueous formic acid) was maintained at 1 mL/min, while the column thermostat temperature was set at 60 °C and the upper pressure limit was fixed at 200 bar. The separations were achieved using a two-step gradient method comprising 62% A and 38% B for 7 min and 70% A and 30% B for 5 min, and the column was brought back to initial conditions in 3 min and equilibrated for 10 min before the next injection. The APCI source of MSD was operated in negative polarity to monitor the selected ions (cohumulone, 347.20; adhumulone and humulone, 361.20; colupulone, 399.30; lupulone and adlupulone, 413.30). The fragmantor was ramped from 50 to 200 for the mass range of 250–500 amu. The spray chamber conditions were as follows: drying gas (nitrogen) temperature, 200 °C; drying gas flow rate, 5 L/min; vaporizer temperature, 325 °C; nebulizer pressure, 60 psig; capillary voltage, 3500 V; and corona current, 25 μA.

The quantification of six hop acids was carried out in the samples using the area under each peak of extracted ion chromatogram (EIC) after establishing a calibration curve by injecting a standardized mixture of hops acids commercially available from Chromadex. The regressions (R^2 values) were established using linear curves, forced origin, and equal weight.

RESULTS AND DISCUSSION

A total of 17 different products obtained locally and examined in this follow-up study include 6 ales, 3 ciders (apple and pear), 6 porters (including stouts), and 2 non-alcoholic lager samples (Table 1). The samples were selected to extend the findings and gaps identified in the first study, particularly because ales represented a broader category of products than initially examined and additional samples were required to screen the wider range of available products. The two porters examined in the first study (NRP 306 and 307) had the highest potential to affect drug safety and efficacy, and additional porters were examined to confirm whether this was specific to those products or was a class effect. Ciders and non-alcoholic beers, which were not examined in the first study, were included here as alcoholic non-hop and non-alcoholic hop controls, respectively.

Dry Weights. There were wide variations in the ale, cider and porter NVSS values with a range from 36.4 mg for an ale (NRP 372) to 87.2 mg for a stout (NRP 380) (Table 1). The nonalcoholic lagers tended to be the lightest products with a mean weight of 48.55 mg/mL whereas the porters were among those with the highest weight of 75.17 mg/mL (range 63.7 to 87.2 mg/mL). The means for the ales and ciders were similar at 59.07 and 59.3 mg/mL but their ranges differed between 36.4 to 84.3 and 49.8 to 77.4 mg/mL, respectively. Guinness (NRP 307, data not shown) from the first study was retested and found to have a similar NVSS dry weight to the first study.

CYP Results. Samples were examined for their potential to affect the CYP-mediated metabolism of the reference standard using either a plate reader or HPLC method (Table 2). The alcohol content of the samples was balanced in the controls.

Table 2. Mean Inhibition (% ± SD) of CYP-Mediated Metabolism by Hops-Containing Products ($n = 2$) in Triplicate, unless Indicated Otherwise[a]

sample	type	3A4 DBF	3A4 testosterone	2D6	2C9
367	ale	27.6 ± 4.11	7.29 ± 1.82	31.6	NA
368	ale	29.2 ± 4.79	13.24 ± 4.53	42.9 ± 13.24	91.6 ± 1.28
369	ale	42.6 ± 3.63	9.31 ± 5.33	58.5 ± 10.35	90.3 ± 1.04
370	ale	43.1 ± 5.04	12.72 ± 2.46	45 ± 1.45	94.5 ± 0.53
371	ale	41.2 ± 4.51	8.12 ± 8.68	49.4	NA
372	ale	26.0 ± 4.99	22.35 ± 9.12	24.9	NA
373	cider	9.4 ± 5.02	−4.15 ± 7.76	4.98 ± 2.02	17.7 ± 4.09
374	cider	8.2 ± 8.33	15.86 ± 5.28	1.4	NA
375	cider	46.9 ± 15.70	−0.33 ± 1.92	13.7 ± 23.70	33.3 ± 4.57
376	stout	65 ± 2.04	25.64 ± 6.11	72.1 ± 11.88	93.8 ± 0.61
377	porter	57.2 ± 1.37	11.96 ± 9.31	66.1 ± 12.68	96.9 ± 1.14
378	stout	90.5 ± 0.28	73.33 ± 12.38	88.9 ± 0.77	99.6 ± 0.17
379	stout	75.7 ± 4.45	19.87 ± 8.18	85 ± 12.54	98.7 ± 0.13
380	stout	87.6 ± 2.48	35.57 ± 8.21	79.7 ± 4.13	99.6 ± 0.35
381	stout	49.5 ± 3.24	12.22 ± 4.45	68.4 ± 25.20	88 ± 2.10
382	lager	4.2 ± 0.23	−4.46 ± 1.71	14.9 ± 4.70	59 ± 3.96
383	lager	19.6 ± 4.12	4.97 ± 1.76	25.4 ± 9.99	76.8 ± 6.36

[a] NA = not analyzed. The ciders were included as non-hops-containing controls, and the <0.5% alcohol lagers were included as non-alcohol controls.

Of the 17 samples examined for the CYP3A4-mediated metabolism of DBF, the highest inhibition was obtained with the 6 porter samples, followed by 1 cider (NRP 376) and then the ales (Table 2). The lowest percent inhibition was obtained with the remaining ciders and the 2 non-alcoholic lagers. The observation that 2 of the 3 ciders had less inhibitory potential partially reflects the different composition of these non-hops-containing products relative to the beers. Because the alcohol content is balanced between test and control samples, alcohol was not expected to affect the findings with these 2 lagers. The results are consistent with the first study,[8] where all lagers tended to have lower inhibitory potential than the other product types. The CYP3A4-mediated metabolism of testosterone to 6-β-hydroxytestosterone was in almost all cases lower, but the trend was generally reflective of the DBF findings, indicating substrate-dependent differences in metabolic specificity.

The highest degree of CYP2D6 inhibition was obtained with the 6 porter samples, followed by the ales (Table 2), consistent with our previous findings.[8] The lowest percent inhibition values were obtained with the lagers and then the ciders. A similar but higher inhibitory effect was noted with CYP2C9.

P-Glycoprotein. Efflux-mediated drug transport can affect drug bioavailability, and this screen were undertaken with the 17 products at two time points (Table 3). The premise for these time points was that the 1 h time course would be representative of an acute response, whereas the longer 18 h values may be indicative of a chronic response. The assay values were normalized to the untreated control cells. Accepting that a difference of less than 20–25% may not be biologically important, 11 of the 17 products may have a minor effect at 1 h, with the 6 exceptions being NRP 372 with increased rhodamine levels and NRP 374, 375, 378, 380, and 381, which had lower values. Of these 6 samples, NRP 374, 375, 378, and 381 values had returned to near that of the controls at 18 h. NRP values had dropped below the control values, whereas those for

5161

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Table 3. Rhodamine 123 Analysis for Samples Incubated for 1 and 18 h with the Hops-Containing Product Relative to Blank Controls[a]

samples	type	Rh123 intake in 1 h (% ± SD)	Rh123 intake in 18 h (% ± SD)
367	ale	116.94 ± 5.96	71.81 ± 9.16
368	ale	87.57 ± 3.11	122.91 ± 10.71
369	ale	81.75 ± 10.48	79.45 ± 3.83
370	ale	88.89 ± 3.70	92.88 ± 2.23
371	ale	107.66 ± 7.05	115.66 ± 9.11
372	ale	160.91 ± 12.84	75.08 ± 4.92
373	cider	83.67 ± 6.12	139.66 ± 11.12
374	cider	61.64 ± 2.60	103.46 ± 3.09
375	cider	69.94 ± 7.08	116.42 ± 6.44
376	stout	104.07 ± 11.29	77.23 ± 5.33
377	porter	80.27 ± 5.99	81.65 ± 8.96
378	stout	50.56 ± 3.56	85.58 ± 10.79
379	stout	82.97 ± 5.41	59.49 ± 8.92
380	stout	63.13 ± 2.29	67.41 ± 1.89
381	stout	66.11 ± 7.47	93.80 ± 3.69
382	lager	76.79 ± 2.32	160.30 ± 9.17
383	lager	83.90 ± 2.12	92.60 ± 4.62

[a] The ciders were included as non-hops-containing controls, and the <0.5% alcohol lagers were included as non-alcohol controls. Values less than 100% suggest induction (and thus more efflux transporter and more activity), and values over 100% suggest inhibition (less active transporter and, hence, more influx of rhodamine).

NRP 380 remained low. At 18 h, 3 of the 11 samples, which had a relatively minor effect, may be biologically important (NRP 373, 379, and 382). Of these, NRP 373 and 382 showed an increase in uptake rhodamine values and NRP 379 showed a decrease. It is noteworthy that 2 ale values dropped about 45 and 86% and 2 (1 cider and 1 porter) values increased about 56 and 83%, respectively, between these two time points. NRP 368 may be noteworthy in that the intake increased by about 35% between the two time points.

Rhodamine values less than 100% indicate that the efflux pump is more active in removing rhodamine from the cell wall membranes, thus reducing intracellular levels. Lower levels, depending upon the time course, can also suggest inductive gene expression with the corresponding increase in functionality. Conversely, values over 100% suggest a less active transporter, allowing for more intracellular rhodamine. As noted above, there may be a time course effect, which in this instance would suggest inhibited gene expression. Additional testing with the determination of gene expression is required however to validate the supposition. Regardless, the findings here demonstrate that changes do occur within these products and contact time and that multiple time points are required to fully elucidate the effect of the product.

This study supports our initial observations[8] that, of the different beer types, porters have the greatest, substrate-dependent, potential to affect CYP2C9- and CYP3A4-mediated biotransformation. The findings with porters are extended to suggest that CYP2D6-mediated biotransformation may also be affected. Some ales may also have an effect on CYP2C9-mediated biotransformation. Any attempt to predict a clinical effect based on extrapolation of in vitro values is fraught with many challenges and should only be undertaken cautiously. This study provides additional mechanistic information to assess any potential risk of these products. Although risk of a serious adverse event resulting from an interaction will depend upon intrinsic and extrinsic factors, including dose and rate of ingestion, in vitro inhibition studies of key metabolic enzymes can serve to evaluate the relative importance of a metabolic pathway.[22] This evaluation into the importance of a pathway is aided by parameters, such as the ratio between the concentration of the drug and the K_i; the caveat is that with multi-constituent hops-containing products or the controls we examined, there is no means of determining a meaningful K_i that would demonstrate the importance of a metabolic pathway. Without an understood a priori threshold for importance, we have for an undiluted product taken a arbitrary value of less than 25% inhibition as being too low to affect drug safety, whereas an inhibition of greater than 75% may suggest that the potential is sufficiently high to warrant further investigation. On this premise, most ciders, lagers, and ales would have a little to limited isozyme and substrate-dependent potential to affect safety and efficacy of health products metabolized by these isozymes, with the main exceptions being some products with greater than 75% inhibition of CYP2C9 and CYP3A4, which may had a higher risk potential to affect the safety and efficacy of health products. Ales and stouts can be consumed in sizable amounts over a relatively short period of time; as with any pharmacological response, the critical factor may be this temporal dose effect. On the basis of the current and previous studies, which confirm the mechanistic potential for hops-containing products to affect drug metabolism and transport, further interaction studies with drugs are warranted to determine the clinical significance of these results with ales and porters.

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5162

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5163

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