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# Plasma and Urine Concentrations of Bioactive Dietary Benzoxazinoids and Their Glucuronidated Conjugates in Rats Fed a Rye Bread-Based Diet

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**ABSTRACT:** Thorough knowledge of the absorption and metabolism of dietary benzoxazinoids is needed to understand their health-promoting effects. In this study, the fates of these bioactive compounds were examined by LC-MS/MS in plasma, urine, and feces after ingesting a daily dose of 4780 ± 68 nmol benzoxazinoids from rye bread using Wistar rats as a model. HBOA-glc (2-$\beta$-D-glucopyranosyloxy-1,4-benzoxazin-3-one) was the predominant benzoxazinoid in the plasma (74 ± 27 nmol/L), followed by DIBOA-glc (2-$\beta$-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one) and HBOA. The total level of benzoxazinoids in the urine was 1176 ± 66 nmol/d, which corresponds to approximately 25% of the total dietary intake. The urinary benzoxazinoid profile differed from that of plasma with HBOA-glc and DIBOA-glc (647 ± 31 and 466 ± 33 nmol/d, respectively) as the major urinary components. The glucuronide conjugates of HBOA and DIBOA were detected in both the plasma and urine. *N*-dehydroxylation was found to be a critical step in the absorption of hydroxamic acids. This unprecedented study will trigger future interest in the biological effects of benzoxazinoids in whole grain rye and wheat diets in humans and other animals.

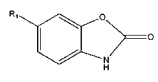
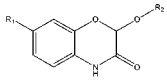
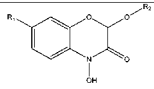
**KEYWORDS:** *benzoxazinoids, absorption, glucuronidation, metabolism, N-dehydroxylation*

## INTRODUCTION

Rye products are important components of the daily whole grain intake in the Scandinavian diet. Epidemiological studies suggest that the consumption of rye bread is associated with a number of beneficial health effects.[1−4] Increasing attention has been given to phytochemicals and their health-promoting roles in whole grain products. An array of benzoxazinoid compounds was recently found to be present in cereal grains and cereal food products.[5−7]

Benzoxazinoids are a group of secondary metabolites that are primarily found in cereals and exhibit potential biological activities including beneficial health effects. These metabolites can be categorized into 3 groups based on their chemical structures: (1) benzoxazolinones, (2) lactams, and (3) hydroxamic acids (Figure 1). Whole grain rye bread, which contains a high benzoxazinoid concentration (~250 $\mu$g/g dry matrix),[6,8] is an important part of the whole grain intake in the Scandinavian diet. Rye grain/bread contains high levels of benzoxazinoids in the form of 2-$\beta$-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one (DIBOA-glc), a double hexose derivative of DIBOA (DIBOA-glc-hex), and smaller amounts of other derivatives.[6,8] Assuming a daily consumption of 50 g of rye bread in Nordic regions,[9−11] the daily total benzoxazinoid intake for a specific brand of whole grain rye bread can be estimated to be higher than 5 mg/d. Nevertheless, very little is known about the absorption and metabolism of these bioactive compounds in mammals.

The use of benzoxazinoid-containing cereal grain products for health-promoting purposes and the medicinal use of a range of benzoxazinoids have already been patented.[5,12] Several in vitro studies have reported the pharmacological effects of benzoxazinoids, including anti-inflammatory, antiallergic,[13,14] and anticarcinogenic activities.[15,16] An epidemiological study has elucidated the weight reduction, appetite suppression, and antidepression activities of benzoxazinoids.[12] In vitro studies raised potential risks for the possible aneugenic and mutagenic effects of the agluconic hydroxamic acids DIBOA and DIMBOA.[17,18] However, agluconic hydroxamic acid is quickly transformed into other metabolites in animal models.[8] A growing interest exists in 6-methoxy-benzoxazolin-2-one (MBOA) due to its structural similarity to melatonin and its effects in stimulating

| Benzoxazolinones | | Lactams | | | Hydroxamic acids | | |
|---|---|---|---|---|---|---|---|
| |  | |  | | |  | |
| | $R_1$ | | $R_1$ | $R_2$ | | $R_1$ | $R_2$ |
| BOA | H | HBOA | H | H | DIBOA | H | H |
| MBOA | $OCH_3$ | HBOA-glc | H | glc | DIBOA-glc | H | glc |
| | | HMBOA | $OCH_3$ | H | DIMBOA | $OCH_3$ | H |
| | | HMBOA-glc | $OCH_3$ | glc | DIMBOA-glc | $OCH_3$ | glc |
| | | HBOA-glc-hex[a] | H | glc | DIBOA-glc-hex[a] | H | glc |

[a]Position and type of this hexose has not been identified.

**Figure 1.** Chemical structures of the most abundant benzoxazinoids quantified.

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the reproductive system.[19] To understand the potential health-promoting properties of benzoxazinoids in vivo, a thorough knowledge of the absorption and metabolism of dietary benzoxazinoids must be obtained.

Various mechanisms are involved in the uptake and metabolism of phytochemicals. Some glucosides are absorbed in their intact forms,[20,21] whereas other glucosides are subject to deglycosylation either by luminal lactase phlorizin hydrolase (LPH) before absorption or by cytosolic $\beta$-glucosidase after active uptake by the sodium-dependent glucose transporter SGLT1.[22−24] The resultant aglycone can be further metabolized leading to the appearance of methyl, sulfate, or glucuronide conjugates in the circulatory system,[25] which are also detected as urine metabolites.[26] The level of bioavailability varies widely for different compounds. A bioavailability value of <0.1% has been reported for both malvidin 3-glucoside and cyanidin 3-glucoside in rats,[21] whereas the bioavailabilities of flavanone and flavonol glucosides range from 1 to 30% in humans.[27−30]

The absorption and metabolism of dietary benzoxazinoids in rats is unknown. For the first time, a detailed study on the uptake and biotransformation of these dietary compounds was recently performed in pigs fed with rye bran-fortified buns.[8] The biotransformations of DIBOA-glc and DIMBOA-glc to their respective aglucones and their subsequent conversion into benzoxazolinones have been reported as allelopathic mechanisms in cereal plants.[31−33] Furthermore, 2-amino-3H-phenoxazin-3-one (APO), a potent antibiotic compound, 2-amino-7-methoxy-3H-phenoxazin-3-one (AMPO), and 2-acetylamino-3H-phenoxazin-3-one (AAPO) are the major transformation products of benzoxazinoids both in the soil and culture medium.[34−36] A similar transformation may also take place in animal models, which could contribute to the beneficial health effects of the parent compounds.

The aim of the present study was to elucidate the absorption, distribution, metabolism, and excretion of bioactive dietary benzoxazinoids in rats, a well-recognized animal model in nutritional and pharmacological studies, fed with a benzoxazinoid-rich, rye bread-based diet.

## MATERIALS AND METHODS

**Chemicals.** HPLC-grade acetonitrile and methanol were obtained from Rathburn (Walkerburn, Scotland). Acetic acid was obtained from Baker (Griesheim, Germany). Inert Ottawa sand (particle size: 20−30 mesh) was purchased from Fisher Scientific (Leicestershire, UK). BOA and MBOA (both with 98% purity) were purchased from Acros Organics (Geel, Belgium). HBOA and HMBOA (purity 98 and 99%, respectively) were synthesized in our laboratory as described in Krogh et al.[37]APO and AAPO (both with 100% purity) were synthesized in our laboratory as described in Gents et al.[34] HBOA-glc (purity 100%), HMBOA-glc (purity 70%), DIBOA (purity 95%), DIMBOA (purity 95%), DIBOA-glc (purity 84%), and DIMBOA-glc (purity 89%) were received as gifts (see acknowledgment section).

**Experimental Design.** The experiment was performed according to protocols approved by the Danish Animal Experiments Inspectorate, Denmark. The experiment was conducted as a parallel study with 2 groups of rats, 12 in each group. Before the analysis, all samples were pooled from 2 rats, resulting in $n = 6$ for each group. The two groups were fed either a semisynthetic control diet (AIN-93G) or a rye bread-based experimental diet supplemented with other nutrients.

**Diets.** AIN-93G (Altromin Spezialfutter GmbH & Co. KG, Lage Germany), which did not contain any detectable benzoxazinoids, was used for both the washout and control diets (Table 1). The experimental test diet consisted of freeze-dried, finely ground rye bread (Multikernerugbrød, Lantmännen Schulstad, Hvidovre, Denmark) with a high benzoxazinoid content and was supplemented with a casein, fat, vitamin, and mineral mixture (Table 1). The food was stored at −18 °C until consumed.

**Table 1. Ingredients (%) of the Rat Diets**

| ingredients | control (AIN-93G) | rye bread-based diet |
|---|---|---|
| freeze-dried rye bread | 0 | 75.45 |
| corn starch | 39.75 | 0 |
| casein - vitamin free | 20.00 | 14.50 |
| maltodextrin | 13.20 | 0 |
| sucrose | 10.00 | 0 |
| soybean oil | 7.00 | 5.00 |
| powdered cellulose | 5.00 | 0 |
| AIN 93G mineral mix | 3.50 | 3.50 |
| AIN 93 vitamin mix | 1.00 | 1.00 |
| L-cysteine | 0.30 | 0.30 |
| choline bitartrate | 0.25 | 0.25 |

**Animals and Feeding.** Twenty-four female Wistar rats (age, 12 wk; body weight, 200−215 g) were purchased from Taconic Europe A/S (Ry, Denmark). The animals were housed in a room with a controlled temperature (22−25 °C) and relative humidity (50−70%) and a 12 h light/dark cycle. Initially, they were kept in group housing cages (4 rats in each, distributed to have a similar mean body weight per cage) and tail-marked with permanent ink for individual identification. All rats had access to the washout diet and water ad libitum for 1 wk. In the second week of the study, half of the rats (12 rats, 3 cages) were fed the rye bread-based diet and the other half continued on the control diet. Next, the rats continued on their respective diets but were housed individually in metabolic cages and given food and water ad libitum for 7 d, with an adaptation period of 3 d and a balance period of 4 d. The food intake during the balance period was monitored individually on a daily basis.

**Sampling.** After a 3 d acclimatization period in individual metabolic cages, urine and feces were collected quantitatively from each rat on a daily basis, frozen, and pooled over the 4 d collection period. The urine was stabilized by adding a saturated ascorbic acid solution (333 g/L) as an antioxidant to the urine collection bottle (1 mL/bottle/d). The feces and urine were stored at −18 °C and pooled from two rats prior to analysis. After 2 wk on the rye bread-based diet, the rats were anesthetized by a subcutaneous injection of 3.75 mg Midazolam, 7.50 mg Fluanisone, and 236 $\mu$g Fentanyl/kg rat. The abdominal cavity was opened, and blood was drawn from the vena cava in heparinized syringes via a Venflon cannula. The blood was transferred to Li-heparin tubes and centrifuged at 2000 g and 4 °C for 10 min. The plasma was pooled (1:1) from two rats and stored at −18 °C until further analysis.

**Extraction of Diet and Fecal Samples.** To quantify the dietary and fecal benzoxazinoid concentrations, extractions of the diets and feces were obtained in duplicate. Freeze-dried diet and fecal samples were ground to particles of less than 0.5 mm prior to analysis. Then, the samples were extracted using an accelerated solvent extraction (ASE) 350 system from Dionex, as previously described.[6]

**Extraction of Plasma and Urine Samples.** Samples were extracted in duplicate. Plasma and urine samples were purified by

solid phase extraction (SPE) using Oasis cartridges (wat094226, 3 cc/60 mg) and $C_8$ cartridges (wat036780, 3 cc/500 mg) respectively as previously described.[8] The samples were stored at −18 °C and diluted with water (1:1) before analysis.

**LC-MS/MS Analysis.** Samples were analyzed by LC-MS/MS using an Agilent 1100 HPLC system coupled with a 3200 Q TRAP mass spectrometer (AB SCIEX, Foster City, CA). Separation of benzoxazinoid compounds was performed using a Synergi Polar RP-80A column (250 mm ×2 mm, 4 μm; Phenomenex, Macclesfield, United Kingdom) with a flow rate of 200 μL/min and an injection volume of 20 μL. All other LC-MS parameters were as previously described.[8] The LC-MS/MS system was controlled by *Analyst Software* (v. 1.5.1) from AB SCIEX.

The identification of the benzoxazinoid glucuronide derivatives was performed using the same instrumentation. Qualitative identification was performed based on the total masses and the similarities between the ESI mass spectrometric fragmentation data and that of the corresponding aglucones and glucosides. The acquisition and identification of these metabolites were also performed using *LightSight Software* (v. 2.2.1) from AB SCIEX.

**Standard Curves.** Standard calibration curves for the 14 compounds listed in Table 2 were prepared by serial dilutions of authentic standard compounds as previously described.[8] The chromatogram peak areas for each of the MRM pairs were measured and used for quantification. The standard curves were applied to a quadratic function with a weight of $^1/_x$ because more data points were at the lower part of the curve ($R > 0.99$). A semiquantitative method was applied to quantify DIBOA-glc-hex and HBOA-glc-hex in the diets and biofluids as previously described[8] due to the unavailability of pure standards.

**Table 2. LC-MS/MS Identification of Benzoxazinoids and Their Phenoxazinone Derivatives and Their Distribution in the Biofluids and Feces of Rats Fed a Rye Bread-based Diet; Blood Samples Were Collected 3 h After Ending Food Intake; Urine and Feces Were Collected on a Daily Basis During the Last 4 Days of the Experimental Period and Pooled From 2 Rats Before Analysis[a]**

| compounds | MW | Q1 ion ($m/z$) | Q3 ion ($m/z$) | $t_R$ (min) | distribution |
|---|---|---|---|---|---|
| BOA | 135.1 | 134.0 | 78.2 | 18.4 | urine, feces |
| MBOA | 165.1 | 164.3 | 149.0 | 23.0 | ND |
| HBOA | 165.1 | 164.0 | 108.0 | 10.7 | plasma, urine, feces |
| HMBOA | 195.2 | 193.9 | 138.2 | 13.3 | urine |
| HBOA-glc | 327.3 | 326.4 | 164.3 | 6.0 | plasma, urine, feces |
| HMBOA-glc | 357.3 | 356.0 | 194.0 | 7.3 | urine |
| HBOA-glc-hex | 489.4 | 488.1 | 164.3 | 4.9 | urine, feces |
| DIBOA | 181.1 | 180.2 | 134.3 | 10.8 | urine, feces |
| DIMBOA | 211.2 | 210.1 | 164.3 | 14.0 | ND |
| DIBOA-glc | 343.3 | 342.0 | 134.0 | 6.6 | plasma, urine, feces |
| DIMBOA-glc | 373.3 | 372.0 | 164.3 | 7.7 | urine |
| DIBOA-glc-hex | 505.4 | 504.1 | 134.0 | 4.9 | urine, feces |
| APO | 212.2 | 213.0 | 185.0 | 36.9 | feces |
| AAPO | 254.2 | 255.0 | 213.5 | 39.7 | urine, feces |

[a]ND, not detectable. For limit of detection, please see Table 3.

**Validation of the Analytical Methods.** The analytical methods were thoroughly validated in our recent publications.[6,8] The concentrations of some benzoxazinoid compounds in some samples were included in the results and discussion even if they were below the limit of quantification (Table 3) because the quantification of concentrations with uncertainties that are higher than the *p*-value defined by EURACHEM is possible, and the error rate in this study's outcomes would be higher if these numbers were omitted than if they were included in the evaluation.[38]

**Data Analysis.** As no benzoxazinoids were detected in either the diet or biofluids of the control rats, no statistical comparisons were made, and all data from the rye bread-fed rats are presented and expressed as the means with their SEM values.

## RESULTS

**Content of Benzoxazinoids in the Diet.** Ten benzoxazinoid compounds were detected and quantified in the rye bread-based diet. The daily intake of total benzoxazinoids from 9.9 ± 0.14 g dry matrix of the rye bread-based diet was measured to be 4780 ± 68 nmol. The composition and daily intake of the individual benzoxazinoids are presented in Table 4. The major benzoxazinoid compound ingested was DIBOA-glc (2243 ± 32 nmol), which comprised 47% of the total daily benzoxazinoid intake and was followed by BOA (925 ± 13 nmol). Additionally, the following compounds were quantified in the given order: DIBOA > DIBOA-glc-hex > HBOA-glc > HBOA > HBOA-glc-hex > DIMBOA-glc.

**Content of Benzoxazinoids in the Plasma.** Three benzoxazinoid compounds (HBOA, HBOA-glc, and DIBOA-glc) were detected in the plasma of the rye bread-fed rats (Figure 2), as outlined in Table 2. HBOA-glc was the predominant compound found in the plasma (74 ± 27 nmol/L) accounting for approximately 68% of the total identified benzoxazinoid compounds.

**Content of Benzoxazinoids and Phenoxazinone Derivatives in the Urine.** The benzoxazinoid compounds that were detected and quantified in the urine samples collected for 4 d from rats fed a rye bread-based diet are presented in Table 4. The mean 24 h urinary excretion of the benzoxazinoid compounds was found to be 1176 ± 66 nmol, which corresponds to 25% of the ingested dose. The main urinary benzoxazinoid compound detected was HBOA-glc (55%), with 647 ± 31 nmol excreted over a 24 h period. DIBOA-glc (466 ± 33 nmol/d) and BOA (40 ± 4 nmol/d) were also detected as major urine compounds excreted over a 24-h period. The total contribution of the other compounds (HBOA > DIBOA > HMBOA-glc > DIBOA-glc-hex > DIMBOA-glc > HBOA-glc-hex > HMBOA) to the overall urinary concentration of benzoxazinoids was minimal (<3%). A negligible amount of AAPO (0.08 ± 0.03 nmol/d) was also excreted in the urine of rats fed a rye bread-based diet.

**Content of Benzoxazinoids and Phenoxazinone Derivatives in the Feces.** Less than 0.7% of the benzoxazinoid intake was detected in the feces (Table 4). HBOA (15.6 ± 1.5 nmol/d) was the predominant benzoxazinoid compound excreted in the feces (50%), followed by small amounts of the other compounds in the following order: DIBOA-glc > DIBOA > BOA > HBOA-glc > DIBOA-glc-hex > HBOA-glc-hex. The benzoxazinoid transformation products APO (0.82 ± 0.02 nmol/d) and AAPO (0.16 ± 0.04 nmol/d) were also excreted in the feces albeit in very small quantities.

Table 3. The Limits of Detection ($L_D$) and Quantification ($L_Q$) For the Analytical Methods of 10 Benzoxazinoid Compounds and Their 2 Possible Derivatives in Wheat Flour, Plasma, Urine, and Feces[6,8]

| | Wheat Flour[a] | | Plasma[b] | | Urine[b] | | Feces[a] | |
|---|---|---|---|---|---|---|---|---|
| analyte | $L_D$ | $L_Q$ | $L_D$ | $L_Q$ | $L_D$ | $L_Q$ | $L_D$ | $L_Q$ |
| BOA | 4.66 | 15.5 | 6 | 20 | 26 | 87 | 4 | 13 |
| MBOA | 0.61 | 2 | 4.9 | 16 | 6.1 | 20 | 1.8 | 6 |
| HBOA | 0.12 | 0.48 | 8.7 | 29 | 10 | 34 | 3.3 | 11 |
| HMBOA | 0.51 | 1.64 | 4.4 | 15 | 13 | 43 | 4.4 | 15 |
| HBOA-glc | 0.4 | 1.31 | 3 | 10 | 7.7 | 26 | 1.3 | 4.4 |
| HMBOA-glc | 0.14 | 0.45 | 2.1 | 7 | 4.8 | 16 | 1.6 | 5.4 |
| DIBOA | 3.37 | 11.3 | 8.8 | 29 | 5.3 | 18 | 6.9 | 23 |
| DIMBOA | 20.3 | 67.7 | 26 | 86 | 2.2 | 7.3 | 6.1 | 20 |
| DIBOA-glc | 0.41 | 1.37 | 3.5 | 12 | 4.1 | 14 | 1.6 | 5.2 |
| DIMBOA-glc | 0.62 | 2.01 | 3.7 | 13 | 9.3 | 31 | 2.6 | 8.7 |
| APO | | | 2 | 6.6 | 1.7 | 5.5 | 4.6 | 16 |
| AAPO | | | 1.1 | 3.8 | 3.8 | 13 | 2 | 6.5 |

[a]nmol $L^{-1}$. [b]nmol $g^{-1}$ dry matrix.

Table 4. Dietary Intake, Urinary Output, Fecal Output, and Recoveries of Benzoxazinoids and Their Phenoxazinone Derivatives in Rats Fed a Rye Bread-based Diet; Urine and Feces Were Collected and Pooled on a Daily Basis During the Last 4 Days of the Experimental Period From 2 Rats Before Analysis[a]

| compounds | intake (nmol/d/rat) | urinary output (nmol/d/rat) (recovery %) | fecal output (nmol/d/rat) (recovery %) |
|---|---|---|---|
| BOA | 925.1 ± 13.3 | 40.1 ± 4.5 (4.3) | 2.6 ± 0.4 (0.3) |
| HBOA | 204.5 ± 3.0 | 9.9 ± 2.7 (4.9) | 15.6 ± 1.5 (7.6) |
| MBOA | 7.2 ± 0.1 | ND | ND |
| HMBOA | ND | 0.2 ± 0.0 | ND |
| HBOA-glc | 331.8 ± 4.8 | 647.6 ± 30.9 (195.7) | 2.7 ± 0.4 (0.8) |
| HBOA-glc-hex | 80.5 ± 1.2 | 0.3 ± 0.1 (0.4) | 0.2 ± 0.0 (0.2) |
| HMBOA-glc | 14.9 ± 0.2 | 2.1 ± 0.2 (13.9) | ND |
| DIBOA | 471.5 ± 6.8 | 7.8 ± 2.6 (1.6) | 3.2 ± 0.8 (0.7) |
| DIBOA-glc | 2243.3 ± 32.3 | 465.9 ± 33.2 (20.8) | 5.5 ± 0.8 (0.3) |
| DIBOA-glc-hex | 462.3 ± 6.7 | 1.2 ± 0.2 (0.3) | 0.2 ± 0.0 (0.0) |
| DIMBOA-glc | 38.9 ± 0.6 | 0.6 ± 0.1 (1.6) | ND |
| APO | ND | ND | 0.8 ± 0.0 |
| AAPO | ND | 0.1 ± 0.0 | 0.2 ± 0.0 |
| Total | 4780.0 ± 68.8 | 1175.9 ± 66.3 (24.7) | 30.9 ± 3.4 (0.7) |

[a]Values are means ± SEM ($n$ = 6). ND, not detectable. For limit of detection, please see Table 3.

**Identification of Glucuronide Compounds in Plasma and Urine.** Two major benzoxazinoid monoglucuronides were characterized by negatively charged ions ($[M\text{-}H]^-$) at $m/z$ values of 340 and 356, with each losing 176 amu upon MS/MS fragmentation, indicating cleavage of the glucuronide conjugate (Figure 3). These compounds were identified as HBOA glucuronide (HBOA-GlcUA) and DIBOA glucuronide (DIBOA-GlcUA), respectively. The diglucuronides of HBOA and DIBOA were not detected in the plasma or urine.

## DISCUSSION

This study revealed, for the first time, the uptake, distribution, metabolism, and excretion of dietary benzoxazinoids in rats fed a rye bread-based diet. The absence of many of the dietary

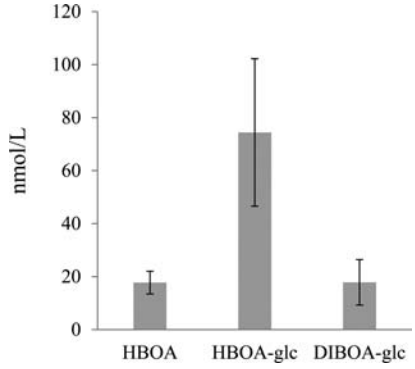


Figure 2. Benzoxazinoid concentration in the plasma of rats fed a rye bread-based diet for two weeks. Three hours after ending food intake, the rats were euthanized and blood samples were collected. The plasma samples of 2 rats were pooled before analysis. Values are the means ± SEM, which are represented by vertical bars ($n$ = 6).

benzoxazinoid compounds in the plasma and their reappearance in the urine (Figure 2 and Table 4) indicated that they were rapidly metabolized in the body and/or quickly eliminated from the bloodstream by the kidneys in their unconverted forms.[8] Nevertheless, considerably higher concentrations of 7 benzoxazinoid compounds were detected in 3 h postprandial blood samples in a similar previous study in pigs.[8] Therefore, the present study indicates the rapid metabolism and/or elimination of benzoxazinoids in rats in contrast to pigs. Moreover, a decrease in the plasma concentrations of HBOA and HBOA-glc at euthanasia after preventing access to food (reflected by error bars in Figure 2, data not shown) also supports the hypothesis of rapid compound elimination from the circulation. These results indicate the need for further studies to determine the accumulation and elimination kinetics of the individual benzoxazinoid compounds in the plasma.

The present study provides little information on the mechanism of the absorption of benzoxazinoids from the gastrointestinal tract and their transformation in the body. HBOA-glc was the most dominant benzoxazinoid compound found in the plasma and urine (Table 4 and Figure 2). The levels of HBOA and HBOA-glc in the urine were 4.8% and 195.7% of the intake levels respectively, whereas the levels of DIBOA and DIBOA-glc in the urine were only 1.6% and 20.8%, respectively. Moreover, the daily intake of DIMBOA-glc was 2 times higher

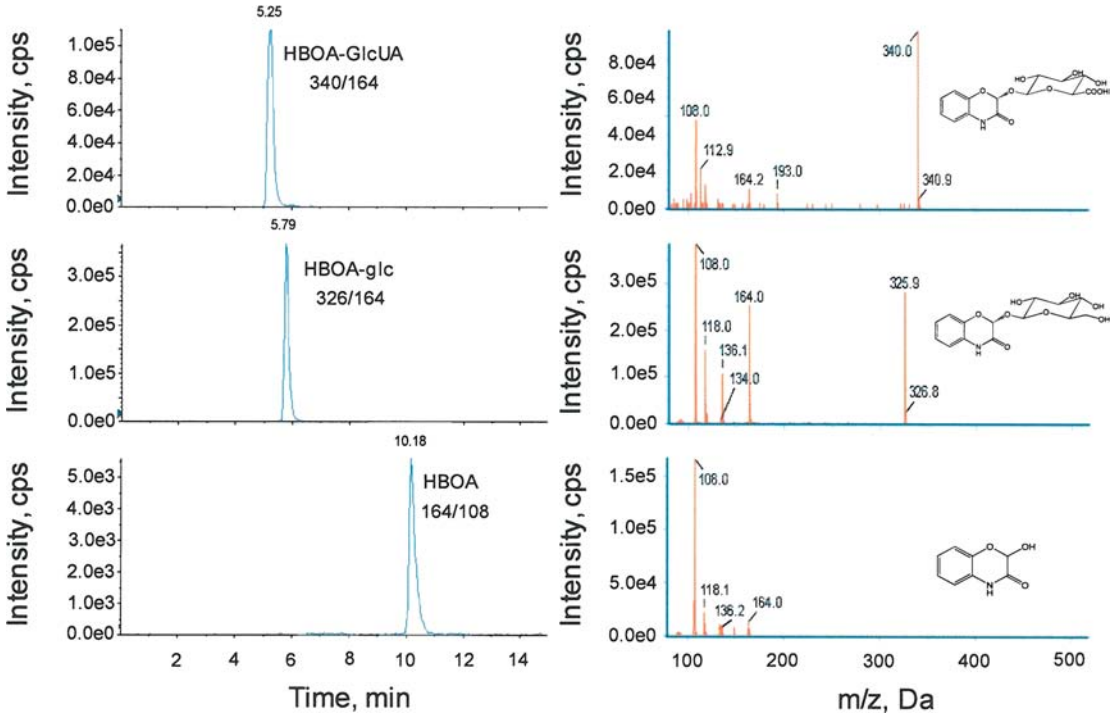


**Figure 3.** Representative extracted ion chromatograms, MS/MS fragments, and structural formulas of HBOA and its derivatives (glucoside and glucuronide) in rat urine following the intake of a benzoxazinoid-rich rye bread-based diet.

than the amount of HMBOA-glc, but the animal's urinary output was nearly 4 times lower (Table 4). The discrepancy between the concentrations of hydroxamic acids and lactams in the diet and biofluids predicts that the structure of the aglycone moiety of benzoxazinoids (Figure 1) is critical for their absorption and metabolism, as suggested for anthocyanins.[21] Moreover, *N*-dehydroxylation of hydroxamic acids (DIBOA, DIBOA-glc, and DIMBOA-glc) into lactams (HBOA, HBOA-glc, and HMBOA-glc, respectively) is a critical mechanism for gastrointestinal absorption and metabolism.[8] This transformation could be caused by microbial activity in the gut. Wheeler et al.[39] reported that certain intestinal microbiota have the capacity to perform *N*-dehydroxylation on some compounds in rats. This finding is in accordance with our previous study performed in pigs.[8] It would be interesting to perform a more detailed study of the role and molecular mechanism of gastrointestinal metabolism in the absorption and/or biotransformation of benzoxazinoids. The absence of the agluconic hydroxamic acid DIBOA in the plasma and the negligible concentration in the urine are positive indications of a low risk of aneugenic and the mutagenic effects in animals, though it was cautioned from recent vitro studies.[17,18]

Dietary HBOA-glc appears to be absorbed from the digestive tract, passed into the circulation and recovered in the urine in its structurally intact glucoside form. This result is in accordance with the direct absorption of cyanidin-3-glucosides in rats.[20] Deglycosylation in the small intestine, as described by Nemeth et al.[40] and Day et al.[22] was not indicated for benzoxazinoid monoglucosides, which instead were directly absorbed and may have been glucuronidated to some extent. The urinary recoveries of the benzoxazinoid monoglucosides HBOA-glc (195.7%), HMBOA-glc (13.8%), and DIBOA-glc (20.8%) in their intact forms were remarkably higher than the urinary recoveries observed for cyanidin 3-glucoside (0.26%) and malvidin 3-glucoside (0.67%).[21] HBOA may have been partially transformed into unidentified compounds by the liver because its urinary concentration was not in the same proportion as the corresponding HBOA-glc or DIBOA-glc (Figure 2 and Table 4).

Although most of the DIBOA-glc was most likely *N*-dehydroxylated prior to absorption, a substantial portion was present in both the plasma and urine as the second most dominant benzoxazinoid, unlike the results obtained in our previous study with pigs.[8] It is likely that the plasma or urinary DIBOA-glc is a derivative of dietary DIBOA-glc-hex and not of dietary DIBOA-glc. The lower concentration and minimal urinary recovery (0.26%) of DIBOA-glc-hex indicates the cleavage of hexose by cytosolic $\beta$-glucosidase in the enterocytes, which releases DIBOA-glc that is actively absorbed into circulation with or without partial glucuronidation. A minor amount of DIBOA-glc-hex was, however, absorbed in its intact form and appeared in urine, as observed for cyanidin-3,5-diglucoside in rats.[20]

This study revealed a high concentration of conjugated or free dietary benzoxazinoids in the biofluids of the rats, with negligible amounts excreted through the feces (Table 4). In addition to their dietary source, the presence of various benzoxazinoids in the feces could arise from the intestinal microbial metabolism of parent benzoxazinoid compounds. Some phytochemicals, such as flavonoids and lignans, are also subject to microbial metabolism in the intestine, with products present in the feces to varying extents.[21,24]

The 26% recovery of the total ingested benzoxazinoids in the rat urine and feces leaves a large proportion of ingested compounds unaccounted for. None of the benzoxazinoids, other than HBOA-glc, displayed a recovery of more than 21% (Table 4). Substantial amounts of some phytochemicals, such as quercetin, are also available to tissues, although in trace amounts, and/or undergo various conjugation metabolism processes in different sites of the body[25,41,42] or are excreted through the lungs as $CO_2$.[43] A recent study by Rosenfeld et al.[12] reported that MBOA was deposited in the antlers of elk maintained on

benzoxazinoid-rich pastures. Interestingly, the daily intake of MBOA (7 nmol) in rats was not detected in any of the examined biofluids, thus indicating its possible deposition in the tissues and/or transformation into other derivatives. Furthermore, MBOA could be present in the biofluids at a concentration that is below the limit of detection due to its negligible intake through the diet. Anyway, it is likely that benzoxazinoids could be partially available to tissues or transformed into other compounds in the rat body that were not monitored in the present study. If MBOA, for example, is delivered to the gut tissue, it may contribute to gut immunity due to its anti-inflammatory effects.[13] This result also necessitates further studies of the tissue distribution of bioactive compounds from rye and wheat, the latter containing mainly methoxylated forms,[44] which could greatly contribute to an understanding of the beneficial effects of these compounds in vivo.

The benzoxazinoid glucuronides HBOA-GlcUA and DIBOA-GlcUA were detected at very low intensities in the plasma but at high intensities in the urine, thus displaying evidence of phase II metabolism. Presumably, these compounds could have been generated through intestinal metabolic processes, as demonstrated for quercetin glucuronides,[25,41] and rapidly eliminated by the kidney. The differences in chromatographic retention times between benzoxazinoid glucuronides and their glucoside or aglucones and the patterns of glucuronide loss during MS/MS fragmentation were similar to those of quercetin metabolites in rat biofluids.[41] The MS/MS analysis of the glucuronide compounds clearly displayed various common fragments of their glucoside or aglucone analogues, confirming their presence (Figure 3). Further investigation of benzoxazinoid compound conjugation may reveal more conjugated benzoxazinoid structures in the future.

In conclusion, this study revealed, for the first time, that dietary benzoxazinoids are highly bioavailable and are absorbed, distributed, metabolized, and excreted in rats. The overall metabolic conversion of benzoxazinoids involves a complex combination of deglycosylation, *N*-dehydroxylation, glucuronidation, and active absorption. The present study is of great importance as an initial step toward understanding the health-promoting effects and biological activities of benzoxazinoids in vivo. The absorption of benzoxazinoid compounds in the body, their health-promoting effects, and the beneficial rye bread-associated health effects reported by epidemiological studies might show a strong correlation. These findings should trigger future interest in the nutraceutical value of these compounds in whole grain rye- and wheat-based diets in humans and other animals.

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### Notes

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## ABBREVIATIONS USED

AAPO, 2-acetylamino-3H-phenoxazin-3-one; APO, 2-amino-3H-phenoxazin-3-one; BOA, 2-benzoxazolinone; DIBOA, 2,4-dihydroxy-1,4-benzoxazin-3-one; DIBOA-glc, 2-$\beta$-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one; DIBOA-glc-hex, double-hexose derivative of DIBOA; DIMBOA, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one; DIMBOA-glc, 2-$\beta$-D-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one; HBOA, 2-hydroxy-1,4-benzoxazin-3-one; HBOA-glc, 2-$\beta$-D-glucopyranosyloxy-1,4-benzoxazin-3-one; HBOA-glc-hex, double-hexose derivative of HBOA; HMBOA, 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one; HMBOA-glc, 2-$\beta$-D-glucopyranosyloxy-7-methoxy-1,4-benzoxazin-3-one; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MBOA, 6-methoxy-benzoxazolin-2-one

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