Journal of Chemical Ecology, Vol. 29, No. 6, June 2003 (© 2003)

GLUCURONURIA IN THE KOALA

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(Received December 3, 2002; accepted February 1, 2003)

Abstract—Glucuronuria is normal in marsupial folivores such as the koala (*Phascolarctos cinereus*), which excretes 2–3 g glucuronic acid daily. Although this has long been attributed to the metabolites of *Eucalyptus* terpenes, we have found that these are mostly excreted in the unconjugated form. We now report on the aglycones that account for most of the glucuronic acid in koala urine. Urine (24 hr) was collected from six male koalas (8.8 ± 0.4 kg, mean ± SE) that were maintained on *E. cephalocarpa* foliage. Urine samples were analyzed by liquid and gas chromatography (LC and GC) coupled with mass spectrometry (MS). Glucuronides were readily identified by LC-MS/MS, which generated characteristic product ions at m/z 113 and 175. From the corresponding parent glucuronide ions, the masses of the aglycones were calculated. Confirmation of identity was by GC-MS after hydrolysis with β-glucuronidase and comparison with standard compounds. Quantitation was by GC. The major non-terpene aglycones were 4-methylcatechol, resorcinol, salicyl alcohol, and two unidentified $C_7H_8O_2$ phenols. Smaller amounts of benzoic acid, benzyl alcohol, orcinol, *p*-cresol, phenol, and phloroglucinol were detected. We have previously reported that terpene metabolites account for about 10% urinary glucuronides in the same koalas fed *E. cephalocarpa*. The present study found that an additional 60% urinary glucuronic acid is conjugated with non-terpene, mainly phenolic,

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0098-0331/03/0600-1465/0 © 2003 Plenum Publishing Corporation

aglycones. It seems likely that these phenolic compounds are present in leaves as glycosides and are chiefly responsible for the glucuronuria in koalas.

Key Words—Glucuronic acid, *Phascolarctos cinereus*, koala, phenolics, *Eucalyptus*, terpenes, detoxification.

INTRODUCTION

Glucuronic acid is not excreted as the free form, but is found in urine conjugated with other molecules called aglycones. Glucuronide conjugates are formed with a variety of endogenous molecules such as steroid hormones, bile acids, and bilirubin, and from a virtually unlimited number of foreign compounds (xenobiotics), including dietary constituents and drugs (Radominska-Pandya et al., 1999; Tukey and Strassburg, 2000; Parkinson, 2001). The role of glucuronidation is to convert lipophilic compounds into highly polar conjugates that can be more readily excreted in urine or bile. Additionally, as glucuronidation usually inactivates drugs and toxins, it is generally considered to be a detoxification reaction, although there are some exceptions where glucuronides are biologically active (Spahn-Langguth and Benet, 1992).

Glucuronides are formed by the reaction of UDP-glucuronic acid with a nucleophilic site on the aglycone molecule, especially the functional groups of phenols, alcohols, or carboxylic acids. The reaction is catalyzed by UDP-glucuronosyltransferase (UGT), of which over 50 forms have been described in vertebrates (Tukey and Strassburg, 2000). UGT activity is present in liver and kidney and also in the epithelia of the respiratory and intestinal tracts where it may have a role in detoxifying xenobiotic substances at the site of contact. As UGT is located in the lumen of the endoplasmic reticulum, substrates must be sufficiently lipophilic to penetrate this membrane barrier before they can be glucuronidated (Radominska-Pandya et al., 1999; Parkinson, 2001). The endoplasmic reticulum is also the site of cytochrome P450 and other phase 1 enzymes, so UGT is well-placed to conjugate the products of functionalization reactions.

The many forms of UGT have different but overlapping substrate specificities (Radominska-Pandya et al., 1999; Tukey and Strassburg, 2000). In general, the UGT1A enzymes glucuronidate a wider range of xenobiotics than do the UGT2B forms. Simple phenols are conjugated most readily by human UGT1A enzymes, except for UGT1A4, especially in the liver and intestine (Tukey and Strassburg, 2000). These authors note that the UGT1A locus is highly conserved in mammals and expressed throughout the gastrointestinal tract, and they propose that the UGT1A enzymes have evolved to metabolize dietary constituents, especially phenols.

Thus, lipophilic dietary xenobiotics enter the cells of the digestive tract where glucuronidation can remove those with suitable nucleophilic sites and allow their excretion in urine or bile. Glucuronides excreted in the bile are liable to hydrolysis

by bacterial β-glucuronidases, but UGT is present at high levels in the colon where it can act to reconjugate the liberated aglycones.

The koala (*Phascolarctos cinereus*) excretes large quantities (2–3 g; 0.9–2.1 mmol/kg/day) of glucuronic acid per day (Hinks and Bolliger, 1957b; Eberhard et al., 1975; Cork et al., 1983). In contrast, humans normally excrete about 1000-fold less on a body weight basis (0.9–1.6 μmol/kg/day; estimated from the data of Lake et al. (1982). The glucuronuria in the koala and other eucalyptus leaf-eating marsupials has been attributed to the detoxification of eucalyptus oils, mixtures of monoterpenes that can comprise up to 10% of the dry leaf mass (Boland et al., 1991). The koala feeds exclusively on eucalyptus leaf, and consequently it is not possible to examine the effect of an artificial diet on glucuronic acid excretion. However, other marsupial folivores, such as the brushtail possum (*Trichosurus vulpecula*), which does not require an exclusively eucalyptus diet, permit manipulative experiments that show the association between the consumption of eucalyptus leaf, or terpenes, and urinary glucuronide excretion (Hinks and Bolliger, 1957a; Dash, 1988). As Dash (1988) reported for the brushtail possum, the ringtail possum (*Pseudocheirus peregrinus*) excreted almost no glucuronic acid when fed a non-eucalyptus artificial diet (McLean et al., 1993). However, after the ringtail was changed to *Eucalyptus radiata* foliage, the excretion of glucuronic acid rose over several days (as leaf consumption increased) to about 1.5 mmol/kg/day (McLean et al., 1993).

However, the excretion of terpene metabolites does not account for all of the glucuronide found in urine of eucalyptus-eating marsupials. In ringtail possum urine, virtually none of the urinary glucuronic acid was linked to terpene metabolites (McLean et al., 1993). The urinary metabolites of two dietary terpenes, *p*-cymene and 1,8-cineole, were found to be mostly or completely unconjugated in the koala, ringtail, brushtail, and greater glider (Boyle et al., 1999; 2000a, 2000b). Recently, we described the urinary metabolites of 1,8-cineole in the koala fed *E. cephalocarpa* leaf (Boyle et al., 2001). The koalas excreted about 2-g glucuronic acid daily (1.1 mmol/kg/day), but only about 10% of this was conjugated with cineole metabolites. Analysis by liquid-chromatography–mass-spectrometry (LC-MS) indicated that the glucuronic acid was conjugated with other small-molecular-weight compounds. The aim of this study was to identify the aglycones in urine that account for the excretion of glucuronic acid in the koala.

METHODS AND MATERIALS

Materials. Standard phenols, alcohols, and acids (Table 1) were obtained from Sigma or Aldrich. Extract of *Helix pomatia* (a mixture of β-glucuronidase 141,000 units/ml and aryl sulfatase 3,950 units/ml) was obtained from Boehringer Mannheim and *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) from Alltech Associates. Other chemicals and solvents were of analytical reagent grade.

TABLE 1. AGLYCONES FOUND AS GLUCURONIDES IN KOALA URINE

					Amount excreted in 24 hr (mean ± SE)	
Formula	MW	Aglycone	RT [a] (min)	Ions monitored[b] (m/z)	Total (μmol/kg)	% Conjugated
$C_7H_8O_2$	124	4-Methylcatechol	5.81	268, 253	357 ± 68	91 ± 1
		Phenol # 1	4.98	254	54 ± 7	86 ± 9
		Phenol # 2	6.07	254, 239	43 ± 5	40 ± 5
		Salicyl alcohol	6.59	268, 253	63 ± 15	87 ± 3
		Orcinol	6.48	268, 253	6 ± 1	100 ± 0
$C_7H_6O_2$	122	Benzoic acid	4.10	194	17 ± 5	100 ± 0
$C_6H_6O_2$	110	Resorcinol	5.83	254	157 ± 82	86 ± 8
C_7H_8O	108	Benzyl alcohol[c]	3.31	180	17 ± 3	100 ± 0
		p-Cresol	3.23	180	4 ± 3	100 ± 0
$C_6H_6O_3$	126	Phloroglucinol	Detected by LC-MS but not quantified			
C_6H_6O	94	Phenol				
				Total non-cineole aglycones	717 ± 128	89.3 ± 16.6
				Total conjugated non-cineole glucuronides	642 ± 119	

[a] GC-MS conditions under Methods and Materials. TMS derivatives were analyzed. The internal standard (3,5-dimethylphenol) had a retention time of 3.95 min and the ion at m/z 194 was monitored.
[b] The first ion was used for quantitation, and the others were used for peak confirmation.
[c] Analyzed by GC-FID using different GC conditions to resolve from the internal standard, which produced the same ions.

Animals and Urine Collection. Six male koalas (*Phascolarctos cinereus*) were used. Animals were maintained on a diet of *Eucalyptus cephalocarpa* and used to investigate the metabolic fate of 1,8-cineole, the major terpene in the leaf of this species. Details of animal care, experimental procedures, and ethical approval have been reported previously (Boyle et al., 2000b, 2001). The koalas (weight 8.8 ± 0.4 kg, mean ± SE) were offered fresh leaves daily, which were picked weekly from a plantation of 12 *E. cephalocarpa* trees. The animals had been feeding on this leaf diet for at least 3 days before urine was collected. Daily leaf consumption was measured (Boyle et al., 2001), enabling the 1,8-cineole intake to be calculated from its concentration in leaf. Urine was collected from koalas for 24 hr and stored at −18°C until analyzed.

Analyses. Glucuronides were identified in urine by MS. Urine samples were diluted (1:10), filtered (0.45 μm), and analyzed by LC-MS, using an LCQ instrument and chromatography conditions described earlier (Boyle et al., 2001). Negative electrospray ionisation (ESI) was used, because positive ESI did not readily detect phenolic glucuronides. ESI conditions were as follows: capillary temperature 300°C, needle voltage 4.5 kV, sheath gas 85 psi, auxillary gas 50 psi and capillary voltage −30 V. Data-dependent MS/MS scans were generated from the most intense ions, and other significant ions were subject to further analysis by MS/MS. Because glucuronides form strong dimer peaks, a series of three scans

was used to detect glucuronides and produce diagnostic product ions. The first was of the total ion current (TIC) from m/z 70–800, showing the major peaks. The second was another full TIC scan from m/z 70–340, excluding the cineole-derived glucuronides ($m/z > 345$) and glucuronide dimer ions ($m/z > 500$). The third scan was the data-dependent MS/MS analysis of the most abundant ion in the second scan. The software automatically selected the largest peak for MS/MS analysis, hence the need to limit the mass range scanned to avoid analysis of large extraneous peaks instead of the peaks of interest. The isolation window was 3 Da and collision energy 23%.

Aglycones were identified, after hydrolysis of glucuronides, using gas-chromatography–mass-spectrometry (GC-MS) and comparison with commercial standards of the phenols, alcohols, and acids likely to be derived from plants (Goodwin and Mercer, 1983). To an aliquot of urine (0.1–0.5 ml, as appropriate) were added 0.25-ml acetate buffer (1.1 M, pH 5.2), 50-μl extract of *Helix pomatia*, and sufficient distilled water to make 1.0 ml. The sample was incubated at 37°C overnight, then acidified to pH 1 with 20-μl 5 M HCl, and vortex mixed with 2.0-ml ethyl acetate for 1 min. The ethyl acetate phase was separated by centrifugation, and 20 μl was mixed with 20 μl BSTFA and heated at 60°C for 5 min to form trimethylsilyl (TMS) derivatives.

GC-MS was carried out with an Hewlett-Packard (HP) 5890 gas chromatograph and 5970B mass selective detector using HP 59970A Chemstation software. The GC was fitted with a 25-m bonded phase capillary column (0.5-μm cyanopropyl film, 0.32-mm i.d.; BP10, Scientific Glass Engineering, Melbourne, Australia). Chromatography conditions were as follows: injection volume 1 μl, split ratio 1:10, He carrier gas 25 psi, injector 215°C, detector 270°C, oven program from 100 to 240°C at 10°C/min. Masses were scanned in the range m/z 40–600 on a 1-sec cycle, and aglycones were identified from their full electron impact (EI) mass spectrum and GC retention time, in comparison with standards.

Aglycones were also quantitated by GC-MS as described earlier, except that internal standard was added to the urine sample (0.5-mg 3,5-dimethylphenol in 0.1-ml water) and the MS used selected ion monitoring (SIM) to ensure adequate selectivity and sensitivity. The following ions were monitored (at 2 cycles/sec): m/z 91, 165, 180 from 1.0 to 3.7 min; m/z 179, 194 from 3.7 to 4.4 min; m/z 108, 126, 139, 254 from 4.4 to 5.5 min; and m/z 239, 253, 254, 268 from 5.5 to 7.0 min. Each aglycone was quantitated using one ion, while others were used for confirmation of identity (Table 1). Calibration standards were prepared by adding suitable quantities of commercial aglycones to blank urine, collected from a brushtail possum that was fed an artificial, non-eucalyptus diet of fruits and vegetables (Boyle et al., 2000a).

Unconjugated metabolites were determined by the same method, except that the hydrolysis step was omitted. Total glucuronic acid was determined colorimetrically (Blumenkrantz and Asboe-Hansen, 1973; Boyle et al., 2000b).

Benzoic acid did not resolve well from the internal standard and, as these compounds also shared the same SIM ions, it was quantitated by flame ionization detection (FID) using different gas chromatography conditions. A Varian 3300 gas chromatograph was fitted with a 30-m Econocap capillary column (0.25-μm SE-54, 0.32-mm i.d.; Alltech Associates). GC conditions were as follows: injection volume 1 μl, split ratio 1:20, carrier He at 9 psi, injector 250°C, detector 290°C, oven held at 100°C for 2 min, then heated at 3°C/min to 135°C, then 30°C/min to 290°C, held for 2 min. Retention times of TMS derivatives were as follows: internal standard 7.65 min, benzoic acid 8.24 min.

RESULTS

Detection of Glucuronides by LC-MS. Figure 1 shows the negative ion mass chromatograms for a sample of koala urine analyzed by LC-MS. The total ion chromatogram (TIC) reflects the sum of all ions in the range m/z 70–800 (upper panel). MS/MS product ion scans can be generated automatically in the LCQ software, using data-dependent selection of the nth most intense ion from a normal

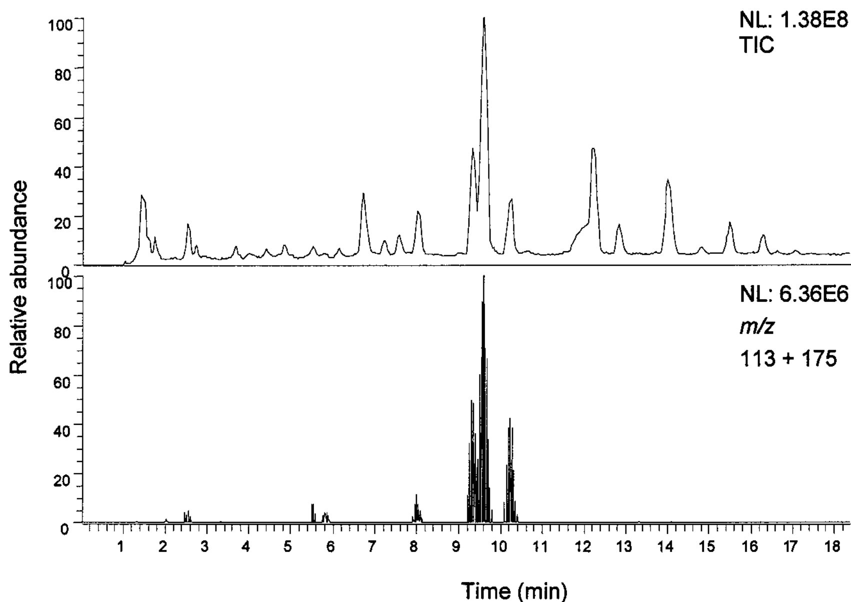

FIG. 1. Chromatograms from LC-MS analysis of koala urine showing non-cineole glucuronide peaks. Upper panel: total ion current (TIC), m/z 70–800. Lower panel: MS/MS product ions monitored at m/z 113 + 175. This trace is interrupted because only every third scan underwent MS/MS analysis. NL is the normalization level, a measure of the height of the largest peak.

scan as the precursor ion. However, most glucuronides formed dimers during the electrospray ionization process producing $[2M\text{-}H]^-$ ions as the most abundant species, which in turn after collisional activation yielded the $[M\text{-}H]^-$ ion corresponding to the original glucuronide. This futile cycle was avoided by including a second full scan, covering just the range m/z 70–340, thus excluding the dimers and ensuring the $[M\text{-}H]^-$ ion was the most abundant. A third scan cycle based on data-dependent selection of precursor ions from the second cycle was included to generate the MS/MS data. Mass chromatograms for the negative ions at m/z 113 and 175 were generated from this (lower panel), as these ions are indicative of glucuronides (Draper et al., 1989; Chen et al., 1998). As the MS/MS data were only produced every third scan, these chromatograms are discontinuous in nature. The full time between consecutive MS/MS scans was 2.4 sec. The molecular weights of the individual parent glucuronides were retrieved from the data, as the data-dependent precursor ion is recorded on each MS/MS spectrum.

Filtering out the higher masses also excluded the cineole glucuronide ions (hydroxy cineole glucuronide has a MW of 346) that appear in the TIC (Figure 1, upper panel) but not the MS/MS chromatogram, which is effectively a selective trace of non-cineole glucuronides (Figure 1, lower panel).

Further evidence of the presence of low-molecular-weight glucuronides is given in Figure 2, which shows selected individual mass chromatograms from LC-MS analysis of the same urine sample. The upper set of chromatograms shows the results for unhydrolyzed urine, and the lower set shows results of the same analyses on the same urine sample after hydrolysis with β-glucuronidase. The parent ions of the glucuronide peaks in Figure 1 were used to generate the corresponding $[M\text{-}H]^-$ ion chromatograms in Figure 2. Since H_2O is lost in the reaction between glucuronic acid and aglycone, the mass of each aglycone can be calculated by subtracting (glucuronic acid – H_2O) (i.e., 176) from the parent molecular weight. For example, the glucuronide $[M\text{-}H]^-$ ion at m/z 299 corresponds to an aglycone mass of 124, possibly methyl catechol, salicyl alcohol, orcinol, or a mixture of these compounds.

Most of the major peaks in the TIC chromatogram were glucuronides (Figure 2). Some were glucuronides of cineole metabolites whose identity was confirmed by positive ES and APCI data, as reported previously (Boyle et al., 2001). The major peaks were non-cineole glucuronides, and their ion chromatograms are displayed in the lower panels of Figure 2. There was also a significant hippuric acid peak, which was confirmed by MS/MS.

MS/MS analysis of each peak confirmed the characteristic glucuronide fragments at m/z 113 and 175 and usually also gave an ion corresponding to the deprotonated aglycone. For example, MS/MS analysis of the major peak shown in the ion chromatogram for m/z 299 (Figure 2) gave the ion m/z 123, corresponding to $C_7H_7O_2$ (Figure 3). Table 1 shows that there are several possible parent aglycones with the formula $C_7H_8O_2$. The glucuronide peaks in Figure 2 also disappeared after hydrolysis with β-glucuronidase (lower panels), although

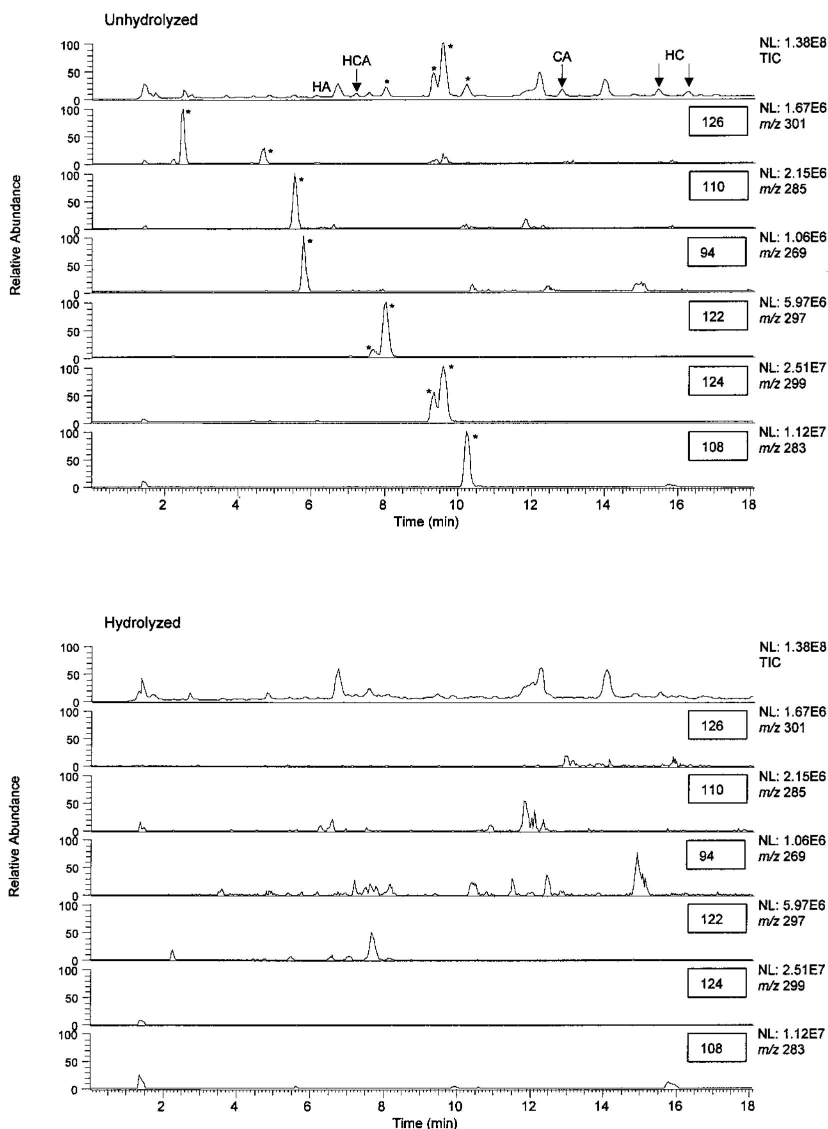

FIG. 2. Chromatograms from LC-MS analysis of koala urine showing TIC and individual mass chromatograms of non-cineole glucuronides. HCA = hydroxycineolic acid glucuronide, CA = cineolic acid glucuronide, HC = hydroxycineole glucuronide (Boyle et al., 2001), HA = hippuric acid, * = non-cineole glucuronide peaks. The upper panels show analyses of unhydrolyzed urine. Under the TIC trace are chromatograms of the $[M\text{-}H]^-$ ions of the non-cineole glucuronide peaks found in Figure 1. The masses of the corresponding aglycones (Table 1) are shown in the boxes. Lower panels: the analyses were repeated on the same sample after hydrolysis, using the same sensitivity settings, showing the absence of glucuronide peaks. NL: normalization level.

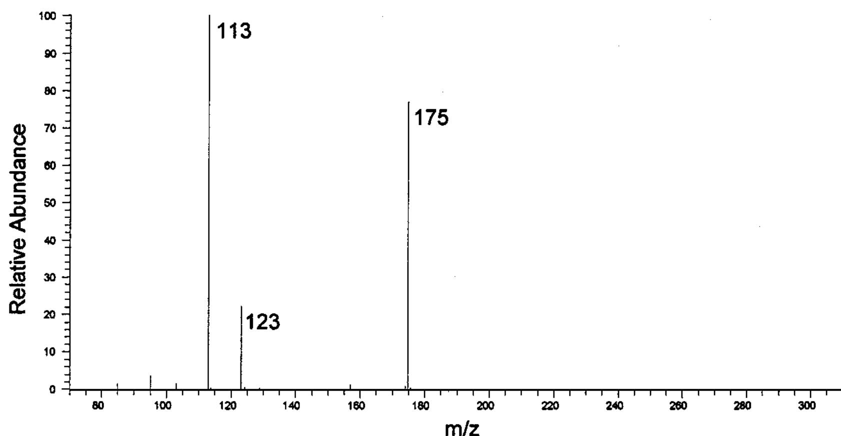

FIG. 3. MS/MS spectrum from $[M\text{-}H]^-$ at m/z 299 showing ions derived from glucuronic acid (m/z 113 and 175) and the deprotonated aglycone (m/z 123, i.e., $C_7H_7O_2$). The possible parent aglycones have the formula $C_7H_8O_2$ and are listed in Table 1.

some extraneous peaks with the same ions were produced. Note that similar sensitivity settings were used before and after hydrolysis, enabling direct comparison between corresponding upper and lower panels. However, the signal strength of each chromatogram depends on that of the formation of each ion and does not indicate the relative quantity of each metabolite.

Identification of Aglycones by GC-MS. The identities of aglycones could not be determined from the LC-MS data alone, so hydrolyzed urine was analyzed by GC-MS and comparison with standard phenols, alcohols, and acids likely to be found in plants. Metabolites were considered to match standards when the retention times and mass spectra were the same (data not shown). Nine metabolites were identified (Table 1), while another two were unknown isomers of methyl dihydroxy benzene (i.e., other than one of the standards). Searches were also made of the TIC chromatograms for possible aglycone molecules, other than the standard compounds selected, but none was found. Not all glucuronide peaks were identified; in particular, those that were present at low levels from the TIC chromatogram were not pursued.

After hydrolysis, the major aglycones in the urine of koalas fed *E. cephalocarpa* were 4-methylcatechol, two unidentified isomeric $C_7H_8O_2$ phenols, salicyl alcohol, and resorcinol (Table 1). No salicylic acid was detected, but there were small amounts of benzoic acid and benzyl alcohol, as well as orcinol and *p*-cresol. The glucuronides of phenol and phloroglucinol were also detected by LC-MS but not quantitated by GC. Phenol was not found by GC-MS and, in

comparison with a phloroglucinol standard, koala urine showed an insignificant peak in the GC-FID chromatogram.

Quantitation. Calibration curves were linear over the range of concentrations measured, with $r^2 \geq 0.99$. There were no interfering peaks present in the samples of blank urine used to prepare calibration standards, either before or after hydrolysis, nor were any of the measured aglycones present.

Comparison of urine before and after hydrolysis showed that, except for one of the unidentified phenols, conjugation was either complete or nearly so for all compounds in Table 1. Although urine was collected on two separate days, only the results from 1 day are presented, as one animal failed to urinate on the other day. However, the amounts of the metabolites excreted, free and conjugated, were not significantly different on the two collection days (paired t tests, $P > 0.05$, 4 df).

Quantitative data on cineole metabolites was taken from a previous study of urine from the same animals collected 2 days later (Boyle et al., 2001). The diet was the same, and the total glucuronic acid excreted (1148 ± 318 μmol/kg, mean $\pm$ SE) was similar to that found in the present study (1077 ± 149 μmol/kg, paired t test $P = 0.823$, 5 df). The aglycones identified were predominantly phenols and unrelated to the terpene metabolites. The total conjugated non-terpenes (642 μmol/kg) accounted for $58.9\% \pm 7.2\%$ total urinary glucuronic acid in the koala.

DISCUSSION

The origin of most of the glucuronic acid in koala urine is attributable to dietary phenols. The phenols, together with small amounts of benzyl alcohol and benzoic acid, accounted for about 60% urinary glucuronides. Previously we have found that cineole-derived glucuronides accounted for 11% of urinary glucuronic acid in the same animals, maintained on the same diet (Boyle et al., 2001). Combining these findings, a total of about 70% of urinary glucuronic acid can be accounted for as phenolic and terpene derived conjugates in the koala maintained on a diet of *E. cephalocarpa* leaf. Although the remaining glucuronic acid is unaccounted for, it seems likely that it is present in conjugation with other, as yet unidentified, aglycones, since virtually no glucuronic acid is excreted when animals are fed a diet free from PSMs.

It seems likely that similar patterns occur in other eucalyptus-leaf-eating marsupials. The brushtail possum formed the same phenolic glucuronides (unpublished data) and, as in the koala, the major proportion of its terpene metabolites were excreted unconjugated. In the ringtail possum and greater glider, virtually none of the terpene metabolites are conjugated, so the urinary glucuronide is likely associated with the same (or similar) leaf-derived phenols.

Low-molecular-weight phenols (such as the aglycones reported here) occur in living cells in combined form with various sugars, mostly glucose (Harborne,

1964; Goodwin and Mercer, 1983). Therefore, the urinary glucuronides are probably derived from glycosides in the leaf. Glycosides which have a hydrophobic domain (e.g., the disaccharide amygdalin) are transported intact across the ileum, and hydrolyzed by a nonspecific hepatic β-glucosidase (LaMarco and Glew, 1986; Gopalan et al., 1992). The liberated aglycone can then be conjugated with glucuronic acid by UGT and UDP-glucuronic acid (Parkinson, 2001). The net result is the conversion of a mono- or disaccharide of glucose or another neutral sugar into the highly ionized glucuronide conjugate, which is excreted more efficiently by the kidney.

This is illustrated by the metabolic fate of salicyl alcohol glucoside (salicin, found in *Salix* and *Populus* spp.) after oral dosage in the brushtail possum (McLean et al., 2001). Some unchanged salicin (10–18% dose) was found in urine, showing that salicin can be absorbed and excreted unchanged. However, most had been hydrolyzed and the major metabolite (56–64%) was the glucuronide conjugate of the aglycone, salicyl alcohol. This is direct evidence of a hydrolysis-reconjugation mechanism for plant glucosides.

Glucuronidation is active in marsupials and appears to be the predominant conjugation reaction for phenols. The fate of [^{14}C] phenol was studied in nine marsupial species, and the major urinary metabolites were glucuronides in the koala (95%) and brushtail possum (90%) (Baudinette et al., 1980). However, a study of the fate of [^{14}C] benzoic acid in seven marsupials found that the glycine conjugate, hippuric acid, was the major metabolite, with benzoyl glucuronide being a minor metabolite in all species (6% dose in the brushtail possum) (Awaluddin and McLean, 1985). Hippuric acid was also the major metabolite of benzoic acid in the koala (Figure 2).

The LC-MS method described here has proven to be an efficient means of detecting glucuronide conjugates in urine. No workup was required, apart from dilution and filtration before liquid chromatography. Negative ESI resulted in satisfactory ionization of glucuronides from all compounds studied: phenols, benzoic acid, and benzyl alcohol as well as the varied terpene metabolites (alcohols, acids, and hydroxy acids) (Boyle et al., 2001). Glucuronide-selective chromatograms can be produced using data-dependent MS/MS scans from the most intense TIC peaks and monitoring for the characteristic ions at m/z 113 and 175, as in Figure 1. The m/z 175 ion corresponds to cleavage at the carbonyl carbon of the glucuronide residue. The mechanism for the formation of the 113 ion is postulated as the further loss of H_2O and CO_2 from the m/z 175 ion (Chen et al., 1998).

The aglycones in this study could not be identified from LC-MS data alone, because of the similarity of their masses and mass spectra. As is usually the case, there were no standards available for the glucuronide conjugates. Therefore, a list of likely compounds was drawn up from known leaf constituents and urinary phenols found in these animals, using the molecular weights that had been found by LC-MS. These standard compounds were analyzed by GC-MS, and their retention times and

mass spectra were compared with those of peaks from hydrolyzed urine extracts. Thus, the combination of LC-MS for detection of glucuronides and GC-MS for identification of aglycones was an efficient means of describing novel glucuronide metabolites. Quantitation was best achieved by analysis of the aglycones before and after hydrolysis to determine the amount excreted free and conjugated.

In the folivorous marsupials that have so far been studied (the koala, brushtail possum, ringtail possum, and greater glider), it appears that glucuronidation is required for xenobiotics that cannot be oxidized sufficiently for rapid excretion (Boyle et al., 1999, 2000a, 2000b, 2001). Thus, phenolic PSMs are mostly conjugated, whereas terpenes are mainly oxidized to metabolites, which are sufficiently polar to be excreted without conjugation. Marsupials use both phase I (oxidative) and phase II (conjugative) metabolic reactions, which target different types of dietary PSMs.

REFERENCES

Awaluddin, A. B. and McLean, S. 1985. Conjugation of benzoic acid in marsupials. *Aus. J. Zool.* 33:693–698.

Baudinette, R. V., Wheldrake, J. F., Hewitt, S., and Hawke, D. 1980. The metabolism of [C^{14}] phenol by native Australian rodents and marsupials. *Aus. J. Zool.* 28:511–520.

Blumenkrantz, N. and Asboe-Hansen, G. 1973. New method for quantitative determination of uronic acids. *Anal. Biochem.* 54:484–489.

Boland, D. J., Brophy, J. J., and House, A. P. N. 1991. Eucalyptus Leaf Oils. Use, Chemistry, Distillation and Marketing. Inkata Press, Melbourne, p. 252.

Boyle, R., McLean, S., and Davies, N. W. 2000a. Biotransformation of 1,8-cineole in the brushtail possum (*Trichosurus vulpecula*). *Xenobiotica* 30:915–932.

Boyle, R., McLean, S., Foley, W., Davies, N. W., Peacock, E. J., and Moore, B. 2001. Metabolites of dietary 1,8-cineole in the male koala (*Phascolarctos cinereus*). *Comp. Biochem. Physiol. C-Toxicol. Pharmacol.* 129:385–395.

Boyle, R., McLean, S., Foley, W. J., and Davies, N. W. 1999. Comparative metabolism of dietary terpene, *p*-cymene, in generalist and specialist folivorous marsupials. *J. Chem. Ecol.* 25:2109–2126.

Boyle, R., McLean, S., Foley, W. J., Moore, B. D., Davies, N. W., and Brandon, S. 2000b. Fate of the dietary terpene, *p*-cymene, in the male koala. *J. Chem. Ecol.* 25:1095–1111.

Chen, X., Zhong, D., Jiang, H., and Gu, J. 1998. Determination of glucuronide metabolitres by electrospray ion-trap mass spectrometry. *Yaoxue Xuebao* 33:849–854.

Cork, S. J., Hume, I. D., and Dawson, T. J. 1983. Digestion and metabolism of a natural foliar diet (*Eucalyptus punctata*) by an arboreal marsupial, the koala (*Phascolarctos cinereus*). *J. Compar. Physiol. B1* 153:181–190.

Dash, J. A. 1988. Effect of dietary terpenes on glucuronic acid excretion and ascorbic acid turnover in the brushtail possum (*Trichosurus vulpecula*). *Comp. Biochem. Physiol.* 89:221–226.

Draper, W. M., Brown, F. R., Bethem, B., and Miille, M. J. 1989. Thermospray mass spectrometry and tandem mass spectrometry of polar, urinary metabolites and metabolic conjugates. *Biomed. Environ. Mass Spectrom.* 18:767–774.

Eberhard, I. H., McNamara, J., Pearse, R. J., and Southwell, I. A. 1975. Ingestion and excretion of *Eucalyptus punctata* D.C. and its essential oil by the koala, *Phascolarctos cinereus* (Goldfuss). *Aus. J. Zool.* 23:169–179.

GOODWIN, T. W. and MERCER, E. I. 1983. Plant phenolics pp. 237-9, 528–566, *in* Introduction to Plant Biochemistry. Pergamon, Oxford.

GOPALAN, V., PASTUSZYN, A., GALEY, W. R., JR., and GLEW, R. H. 1992. Exolytic hydrolysis of toxic plant glucosides by guinea pig liver cytosolic beta-glucosidase. *J. Biol. Chem.* 267:14027–14032.

HARBORNE, J. B. 1964. Phenolic Glycosides and their natural distribution, pp. 129–169, *in* J. B. Harbone (ed.) Biochemistry of Phenolic Compounds. Academic Press, New York.

HINKS, N. T. and BOLLIGER, A. 1957a. Glucuronuria in a herbivorous marsupial *Trichosurus vulpecula*. *Aus. J. Exp. Biol.* 35:37–44.

HINKS, N. T. and BOLLIGER, A. 1957b. Glucuronuria in marsupials. *Aus. J. Sci.* 19:228–229.

LAKE, B. G., LONGLAND, R. C., HARRIS, R. A., and GANGOLLI, S. D. 1982. The excretion of metabolites of the D-glucuronic acid pathway in human urine. Effect of phenobarbitone administration. *Xenobiotica* 12:241–247.

LAMARCO, K. L. and GLEW, R. H. 1986. Hydrolysis of a naturally occurring beta-glucoside by a broad-specificity beta-glucosidase from liver. *Biochem. J.* 237:469–476.

MCLEAN, S., FOLEY, W. J., DAVIES, N. W., BRANDON, S., DUO, L., and BLACKMAN, A. J. 1993. Metabolic fate of dietary terpenes from *Eucalyptus radiata* in common ringtail possum (*Pseudocheirus peregrinus*). *J. Chem. Ecol.* 19:1625–1643.

MCLEAN, S., PASS, G. J., FOLEY, W. J., BRANDON, S., and DAVIES, N. W. 2001. Does excretion of secondary metabolites always involve a measurable metabolic cost? Fate of plant antifeedant salicin in common brushtail possum, *Trichosurus vulpecula*. *J. Chem. Ecol.* 27:1077–1089.

PARKINSON, A. 2001. Biotransformation of xenobiotics, pp. 133–224, *in* C. D. Klaasen (ed.). Casarett and Doull's Toxicology: The Basic Science of Poisons. McGraw-Hill, New York.

RADOMINSKA-PANDYA, A., CZERNIK, P. J., LITTLE, J. M., BATTAGLIA, E., and MACKENZIE, P. I. 1999. Structural and functional studies of UDP-glucuronosyltransferases. *Drug Metab. Rev.* 31:817–899.

SPAHN-LANGGUTH, H. and BENET, L. Z. 1992. Acyl glucuronides revisited: Is the glucuronidation process a toxification as well as a detoxification mechanism? *Drug Metab. Rev.* 24:5–48.

TUKEY, R. H. and STRASSBURG, C. P. 2000. Human UDP-glucuronosyltransferases: Metabolism, expression, and disease. *Annu. Rev. Pharmacol. Toxicol.* 40:581–616.