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IDENTIFICATION OF SOME HUMAN URINARY METABOLITES OF THE INTOXICATING BEVERAGE KAVA

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SUMMARY

Methane chemical ionization (CI) gas chromatography–mass spectrometry (GC–MS) has been used to identify some of the human urinary metabolites of the kava lactones following ingestion of kava prepared by the traditional method of aqueous extraction of *Piper methysticum*. All seven major, and several minor, kava lactones were identified in human urine. Observed metabolic transformations include the reduction of the 3,4-double bond and/or demethylation of the 4-methoxyl group of the α-pyrone ring system. Demethylation of the 12-methoxy substituent in yangonin (or alternatively hydroxylation at C-12 of desmethoxyyangonin) was also recognised. This product was isolated by high-performance liquid chromatographic analysis of crude urine extracts and characterised by methane CI GC–MS. In contrast to the situation prevailing in the rat no dihydroxylated metabolites of the kava lactones, or products from ring opening of the 2-pyrone ring system, were identifed in human urine. GC–MS analysis of urine can be readily utilised to determine whether donors have recently consumed kava.

INTRODUCTION

Kava, the intoxicating beverage of the Pacific Islands[1,2] has recently become a drug of abuse amongst some of the aboriginal communities of the Northern Territory of Australia[3–6]. *Piper methysticum* is imported mainly from Fiji and Vanuatu and aqueous extraction of the commercially dried plant material is used for

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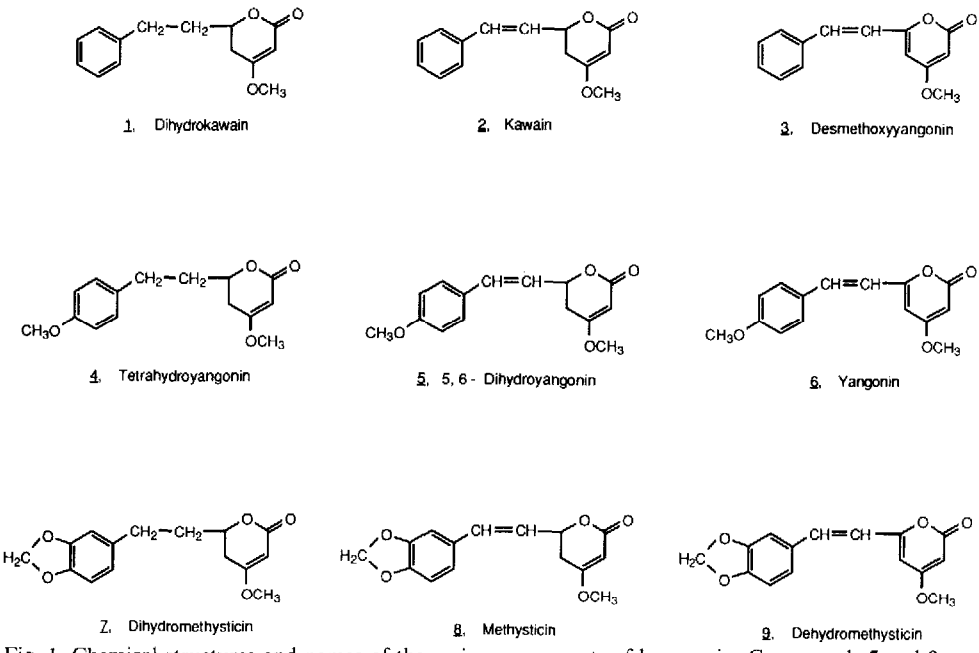


Fig. 1. Chemical structures and names of the major components of kava resin. Compounds **5** and **9** are present in only trace amounts.

the preparation of kava. The chemical structures of the lipid constituents of kava, often referred to as kava resin, and conveniently isolated by solvent extraction of *Piper methysticum*, have been defined (see Fig. 1)[1,2,7,8]. We have recently described[7,8] the application of gas chromatography–mass spectrometry (GC–MS) to the identification of known major, and several previously undetected minor constituents, of this resin.

One report has appeared[9] on the metabolism of kava lactones in the rat. This involved GC–MS analysis of urine from rats given, by stomach tube, an aqueous suspension of selected pure kava lactones. In the case of dihydrokawain (**1**) and kawain (**2**) the major urinary metabolite resulted from *p*-hydroxylation of the phenyl ring accompanied by smaller amounts of a further hydroxylation product. In addition minor metabolites arising from ring opening of the unsaturated 4-methoxy-2-pyrone ring system were recognised[9]. In contrast methysticin (**8**) produced only trace amounts of urinary metabolites resulting from rupture of the methylenedioxy ring system to a catechol structure, with and without concommitant reduction of the 7,8-double bond. Yangonin (**6**) and its 7,8-dihydro analogue yielded no identifiable urinary metabolites from rupture of the 2-pyrone ring system but were demethylated at C-12 to a phenol which was the precursor of other hydroxylated metabolites[9].

In addition to these metabolic transformations rat urine also contained unchanged kava lactones, which in the case of dihydrokawain (**1**), amounted to a 48% recovery of an administered dose after 48 h[9]. We now wish to report on the complex mixture of metabolites, and unchanged kava lactones, which are excreted in human urine after ingestion of kava prepared by the traditional method of water extraction of commercial *Piper methysticum*.

EXPERIMENTAL

Preparation of kava

Commercial *Piper methysticum* (450 g), was immersed (muslin bag) in water (3 l) at room temperature and after squeezing (5 min) "kava" was obtained. The beverage was consumed by healthy male subjects in about 100-ml aliquots. Typically 1 l was consumed in a period of about 1 h before sleeping and no physiological effects were apparent after consumption of this dose. Urine samples were collected before sleep and again on rising in the morning.

Urine sample work-up for direct GC–MS

All solvents were distilled prior to use. Urine (100 ml) was acidified (pH 1) with 2 *M* hydrochloric acid and extracted with chloroform (3 × 30 ml), centrifuged to break persistent emulsions, and the combined organic phase washed with 5% aqueous sodium carbonate (2 × 20 ml), water (20 ml) and then the chloroform layer was dried over anhydrous sodium sulphate. Filtration and removal of the chloroform *in vacuo* provided a residue 10% of which was derivatised in a Reactivial with bis(trimethylsilyl)trifluoroacetamide (BSTFA) (50 μl) (Pierce, Rockford, IL, U.S.A.) at 80°C for 30 min and aliquots (1–2 μl) used for GC–MS analysis.

GC–MS

MS instrumentation consisted of a Finnigan Model 3200 CI GC–MS system interfaced to a Finnigan-Incos Model 2300 data system. It was operated under the conditions previously described[7]. GC separations were made with a quartz BP-10 capillary column (25 m × 0.3 mm I.D.; helium flow, 1 ml/min; Scientific Glass Engineering, Melbourne, Australia). Samples were injected at an initial oven temperature of 100°C which was temperature programmed at 6°C/min to a final temperature of 300°C. The CI mass spectrometer was scanned in 1 s between *m/z* 60 and 460 during these analyses. Methane served as the CI reactant gas at an ion source pressure of 1 Torr.

HPLC

Gradient purifications were carried out with a Waters Model 510 pump fitted with a U6K injector, a Waters M45 pump and a Model 660 solvent programmer. Reversed-phase purifications utilized an Econosil C_{18} column (250 mm × 4.6 mm I.D., particle size 5 μm) (Alltech Assoc., Homebush, Australia). The detection system included a Hewlett Packard 1040A diodearray interfaced with a HP 9000 series 300 computer and a HP 9133 disc drive. Chromatograms were recorded at 220, 260 and 360 nm with a bandwidth of 4 nm. The reference wavelength was set at 550 nm with a bandwidth of 100 nm. UV spectra and chromatograms were hard-copied using either a HP Model 7470A plotter or a HP Thinkjet printer.

Urine extraction and HPLC separation

Urine (50 ml) was extracted with chloroform (3 × 20 ml) as described above and the neutral residue dissolved in methanol (0.5 ml) which was then fractionated into two separate injections under the above HPLC conditions. A linear gradient was used, programmed from 10% methanol in 0.2% aqueous acetic acid to 100% methanol

during 30 min. The fraction eluting between 28 and 29 min was collected and evaporated to dryness for characterisation by methane CI MS.

The chloroform extract from the urine of a second volunteer was chromatographed using the same column but a different mobile phase consisting of 45% acetonitrile in 0.2% aqueous acetic acid. The eluent from a single injection of this extract was collected between retention times of 2 and 10 min and evaporated to dryness. This residue in methanol was re-injected under the same conditions. The peak displaying the *trans*-yangonin chromophore (Fig. 3) was trapped (retention time 8–9 min) and the solvent removed. This material was then characterised by methane CI MS and shown to be identical with that isolated by high-performance liquid chromatography (HPLC) using the methanol based solvent system.

*Hydrogenation of dihydrokawain to 3,4,7,8-tetrahydrokawain (**10a**)*

Dihydrokawain (10 mg) in ethyl acetate (10 ml) was hydrogenated at 55–60°C for 5 h over a 10% Pd/C catalyst (5 mg). Solvent was added as necessary to maintain the reaction mixture at a volume of between 5 and 10 ml. The catalyst was filtered off and the residue shown by methane CI MS to have an $[MH]^+$ at *m/z* 235 and its mass spectrum and retention time was similar to that recorded for **10a** in Table II and Fig. 2.

*Hydrogenation of tetrahydroyangonin (**4**)*

In a similar experiment **4** was hydrogenated to 3,4,5,6,7,8-hexahydroyangonin identified by its methane CI mass spectrum[a] as **10b**: $[MH]^+$ *m/z* 265 (55%), *m/z* 247 (15%), *m/z* 233 (40%), *m/z* 215 (11%), *m/z* 187 (4%), *m/z* 173 (6%), *m/z* 147 (28%),

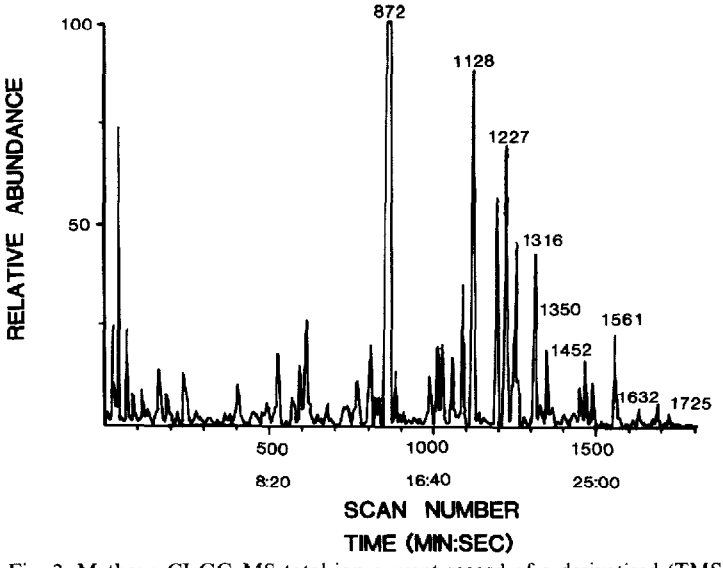


Fig. 2. Methane CI GC–MS total ion current record of a derivatised (TMS) urine extract after human consumption of kava. The GC conditions were as follows: BP-10 column (25 m × 0.3 mm O.D.) which was temperature programmed from 100 to 300°C at 6°C min^{-1} 1.5 min after sample injection.

[a] Unless indicated to the contrary all $[MH]^+$ ions were accompanied by the methane adduct species $[M+C_2H_5]^+$ and $[M+C_3H_5]^+$, respectively.

m/z 121 (100%) and *m/z* 103 (8%). We were unable to detect by methane CI GC–MS the presence of this compound in extracts of kava urine.

RESULTS AND DISCUSSION

Caffeine provided a focal point (scan number 872 in Fig. 2) in the GC–MS analysis of TMS derivatised residues from human urine after consumption of kava. All the identified kava resin components and their metabolites had longer GC retention times than caffeine. The major unchanged kava lactones whose structures are summarised in Fig. 1 (**1–4**, **6–8**) were readily identified from their methane CI mass spectra[7] and their identity and scan number in Fig. 2 are recorded in Table I.

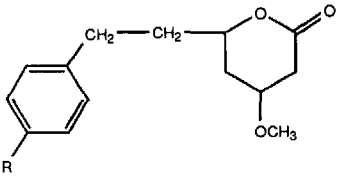
TABLE I

IDENTIFICATION IN FIG. 2 OF UNCHANGED KAVA LACTONES PRESENT IN HUMAN URINE AFTER DRINKING KAVA

Compound	*Scan number*	*$[MH]^+$ m/z*
Dihydrokawain (**1**)	1195	233
Kawain (**2**)	1316	231
Desmethoxyyangonin (**3**)	1350	229
Tetrahydroyangonin (**4**)	1452	263
Dihydromethysticin (**7**)	1561	277
11-Methoxytetrahydroyangonin	1612	293
Yangonin (**6**)	1632	259
Methysticin (**8**)[a]	1645	275
Dehydromethysticin (**9**)	1725	273

[a] Methysticin (**8**) was not detected in Fig. 2. Other analyses of kava urine extracts did give a positive identification for **8** at this retention time (immediately following yangonin). The GC column must be relatively new and free of decomposition products to transmit methysticin to the MS ion source[7].

The first identified kava metabolite eluted at scan number 1060 (Fig. 2) and had a protonated molecular ion ($[MH]^+$) at *m/z* 235 and its mass spectrum is summarised in Table II. This metabolite corresponds to saturation of the 3,4-double bond of dihydrokawain (**1**) so that the metabolite would be **10a**. This was confirmed by synthesis of **10a** from catalytic hydrogenation of dihydrokawain. We did not detect analogues of **10a** corresponding to methoxyl (**10b**, $[MH]^+$, *m/z* 265) or methylenedioxy ($[MH]^+$, *m/z* 279) substitution of the phenyl ring [*i.e.*, metabolites of tetrahydroyangonin (**4**) and dihydromethysticin (**7**) respectively].



10a, R = H, $[MH]^+$, *m/z* 235
10b, R = OCH_3, $[MH]^+$, *m/z* 265

TABLE II

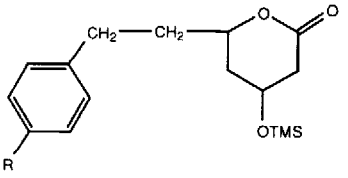
METHANE CI MASS SPECTRA OF THE TMS DERIVATIVES OF KAVA LACTONE METABOLITES IDENTIFIED IN HUMAN URINE

Scan No.	*Structure*	*$[MH]^+$ m/z*	*Mass spectrum*[a]
1060	**10a**	235	235,100; 217,27; 203,70; 185,65; 157,17; 143,68; 117,23; 103,27; 91,5
1092, 1129[b]	**11a**	293	293,18; 277,6; 275,10; 243,2; 233,3; 231,7; 203,65; 185,69; 161,55; 157,22; 143,100; 117,20; 91,16
1365	**11b**	323	323,6; 251,12; 233,35; 215,11; 191,10; 173,8; 161,22; 147,15; 121,100; 75,7; 73,9
1436, 1470[b]	**11c**	337	337,6; 336,4; 321,7; 275,6; 247,68; 229,24; 205,12; 187,35; 161,95; 135,100; 75,18; 73,13
1227, 1255[b]	**12a**	291	291,1; 275,1; 201,70; 189,12; 183,20; 161,100; 157,9; 155,28; 145,9; 143,7; 141,12; 131,6; 117,11; 101,10; 91,23; 75,28; 73,30
1332	**12b**	321	321, 100; 305,12; 231,48; 215,8; 207,12; 121,40; 91,8; 75,4; 73,8
1677	**13**	317	317,100; 301,9; 245,6; 125,25

[a] *m/z*, % relative intensity.
[b] Relative intensity variations in *m/z* values between each isomeric pair was within 10%.

The next urinary metabolites to be recognised eluted (Fig. 2) at scans 1092 and 1128 and they had very similar methane CI mass spectra with $[MH]^+$ ions of *m/z* 293 (Table II). These products formally correspond to structure **11a** and may be C_4 epimers.

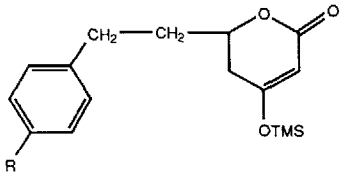
Two methylenedioxy substituted metabolites with identical mass spectra ($[MH]^+$, *m/z* 337) appear at scan numbers 1436 and 1470 respectively in Fig. 2 and structure **11c** is consistent with the mass spectral evidence (Table II). Presumably these metabolites **11a** + **11c** would be derived from either dihydrokawain (**1**), or kawain (**2**), and either dihydromethysticin (**7**) or methysticin (**8**), respectively. Interestingly only one isomer of the methoxylated version, **11b** ($[MH^+$, *m/z* 323) was identified (Table II, scan number 1365 in Fig. 2) in all the urine samples investigated and presumably this arose from metabolism of the tetrahydroyangonin (**4**) content of kava.



11a, R = H, $[MH]^+$, *m/z* 293
11b, R = *p*-OCH_3, $[MH]^+$, *m/z* 323
11c, R = 11,12-O–CH_2–O–, $[MH]^+$, *m/z* 337

Another metabolite ($[MH]^+$, *m/z* 291) was detected as two isomers with similar methane CI mass spectra at scan numbers 1223 and 1255 (Fig. 2) and formally correspond to the demethylation product (**12a**) of dihydrokawain (**1**). Tetramethylsilyl

(TMS) derivatisation of the 1,3-diketo metabolite would be expected to yield[10] a mixture of 2- and 4-OTMS structures. The methoxylated analogue (**12b**) [presumably the metabolite of tetrahydroyangonin (**4**)] elutes (Fig. 2) at scan number 1332 ($[MH]^+$, m/z 321). The anticipated second isomer of **12b** may co-elute with desmethoxyyangonin (**3**) around scan number 1350 but the relatively large amount of **3** did not allow positive clarification of this point. Only weak responses were detected corresponding to the methylenedioxy metabolite (**12c**) ($[MH]^+$, m/z 335) in some urine analysis but insufficient ion current was available for definitive identification.



12a, R = H, $[MH]^+$, m/z 291
12b, R = p-OCH_3, $[MH]^+$, m/z 321
12c, R = 11,12-O–CH_2–O–, $[MH]^+$, m/z 335

Scan 1677 in Fig. 2 is a kava metabolite ($[MH]^+$ at m/z 317) which corresponds to the product of demethylation (**13**) of the 12-methoxyl group of yangonin (**6**) or, alternatively, from hydroxylation at C-12 of desmethoxyyangonin (**3**). This metabolite was isolated from crude urine extracts by HPLC using multivariate UV detection. The UV spectrum of the material collected from HPLC analysis was very similar to yangonin (Fig. 3) and its properties are consistent with its assignment as 12-desmethylyangonin (**13**). This was confirmed from solid probe methane CI-MS which yielded a single product, {$[MH]^+$, m/z 245 (base peak)} with a fragment of m/z 125 (15% relative abundance). This fragmentation behaviour is similar to yangonin[7] and the fragment at m/z 125 confirms the presence of the 2-pyrone ring system in the metabolite[7]. Derivatisation (TMS) of the product collected from HPLC afforded a compound ($[MH]^+$, m/z 317) whose methane CI GC mass spectrum was identical to that obtained (Fig. 2) from direct TMS derivatisation of the crude urine extract.

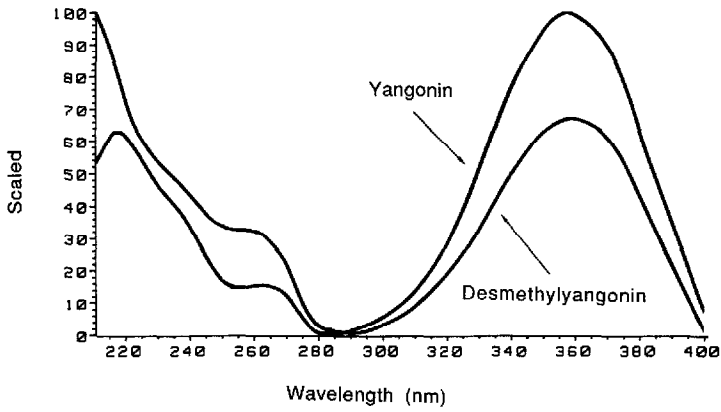
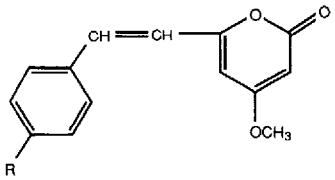


Fig. 3. UV spectra of yangonin (**6**) and a metabolite isolated by HPLC and identified as 12-desmethylyangonin (**13**).

C-12-Desmethylyangonin (**13**) was identified[9] as a metabolite of yangonin (**6**) in rat urine.



13, R = OH, $[MH]^+$, *m/z* 245
R = OTMS, $[MH]^+$, *m/z* 317

In contrast to the situation pertaining to the metabolism by rodents of kava lactones[9] we did not detect in human urine products resulting from opening of the 2-pyrone ring of the kava lactones (Fig. 1). These products would have been removed in the 5% sodium carbonate wash employed in the urine work-up. Acidification of this aqueous extract and solvent extract isolated a crude acidic fraction which was then derivatised as its TMS ester/ether analogues. GC–MS of this derivatised fraction failed to identify any mass spectra compatible with pyrone ring opened metabolites of the kava lactones. Other metabolites of the kava lactones are present in human urine but their identity could not be determined on the evidence currently available.

This methane CI GC–MS analysis was used to screen over 80 urine samples collected from aboriginal donors in the Northern Territory. We were able to define those individuals who had not taken kava (no kava lactones or metabolites detected), those who had previously, but not recently, consumed kava (trace to small amounts of marker lactones observed) and those who had recently consumed a quantity of kava (identification of relatively large amounts of kava lactones and there metabolites). Our analysis of these samples correlated with the results of a questionnaire completed by the urine donors regarding their recent use of kava. This work demonstrated that it is relatively easy to define by urine analysis those individuals who have consumed kava and this may be of interest to forensic analysts.

ACKNOWLEDGEMENTS

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