Uptake into Mouse Brain of Four Compounds Present in the Psychoactive Beverage Kava

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Received April 25, 1988, from the *School of Physiology and Pharmacology, and ‡Biomedical Mass Spectrometry Unit, The University of New South Wales, P.O. Box 1, Kensington, N.S.W., Australia, 2033. Accepted for publication July 27, 1988.

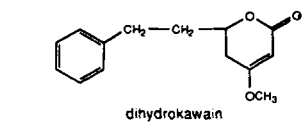
Abstract □ A technique using gas chromatography–mass spectrometry and deuterated internal standards is described for the quantitation in brain tissue of four constituents of the intoxicating beverage kava. Dihydrokawain, kawain, desmethoxyyangonin, and yangonin were administered ip to mice at a dosage of 100 mg/kg. At specific time intervals (5, 15, 30, and 45 min), the mice were sacrificed and the brain concentrations of these four compounds determined. After 5 min, dihydrokawain and kawain attained maximum concentrations of 64.7 ± 13.1 and 29.3 ± 0.8 ng/mg wet brain tissue, respectively, and were rapidly eliminated. In contrast, desmethoxyyangonin and yangonin had poorly defined maxima corresponding to concentrations of 10.4 ± 1.5 and 1.2 ± 0.3 ng/mg wet brain tissue, respectively, and these compounds were more slowly eliminated from brain tissue. When crude kava resin was administered ip at a dosage of 120 mg/kg, the concentration in brain of kawain and yangonin markedly increased (2 and 20 times, respectively) relative to the values measured from their individual injection. In contrast, dihydrokawain and desmethoxyyangonin, after the administration of crude resin, remained at the percentage incorporation into brain tissue established for their individual ip injection.

Kava is the intoxicating beverage of the South Pacific Islands and is prepared from aqueous extraction of the roots of the shrub *Piper methysticum*. Drinking kava is reported to produce a state of quiet relaxation differing from that produced by alcohol in that the former induces a placid tranquility.[1] The peoples of the South Pacific have described the diuretic, sudorific, analgesic, and antipyretic properties of kava, and it has been utilized in the treatment of venereal disease, gout, rheumatism, diarrhea, and asthma.[2] The major physiological action in humans is consistently reported as a mild, centrally acting relaxant property which induces a generalized muscle relaxation and, ultimately, a deep natural sleep.[3] A minor property of kava is its local anesthetic properties which are experienced as a numbing of the mucous membranes of the mouth and tongue when the beverage is consumed.[3]

Kava was introduced from Fiji in 1983 to aboriginal communities of Northern Australia and rapidly became a drug of abuse[4] because of a lack of ceremonial or traditional restraints controlling its use. Estimates for individual consumption have ranged as high as fifty times the amount of kava habitually consumed in the Pacific Islands and the epidemic of kava abuse has become a serious social problem in regions of Northern Australia.[5]

The seven major constituents of kava resin, the product of organic solvent extraction of the roots of *P. methysticum*, have been reviewed[6] and their chemical structures are shown below. Recently, we have used gas chromatography–mass spectrometry (GC–MS) to analyze kava resin and have identified several new minor components.[7,8]

One known effect of continual kava consumption is a skin affliction termed kani which is characterized by light and dark bands on the skin, which becomes rough and scaly.[9] Because of the lack of knowledge regarding the effects of prolonged high dosage consumption of kava, we have com-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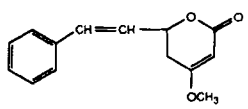
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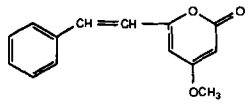
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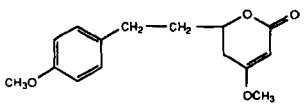
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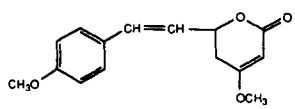
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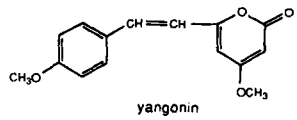
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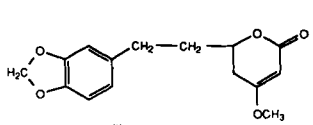
6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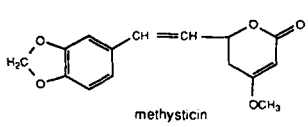
7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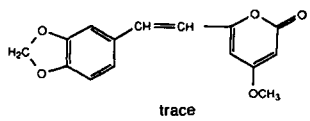
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menced a study of the pharmacology of four of these psychoactive constituents of kava resin and, as a consequence, we have developed a sensitive assay for their specific detection. In this report we wish to describe the uptake into mouse brain tissue of dihydrokawain (1), kawain (2), desmethoxyyangonin (3), and yangonin (6), all of which are known to be active as anticonvulsants when tested in mice against maximal electroshock seizures.[10,11] With a knowledge of the brain concentrations of these psychoactive compounds, animal behavioral experiments can be designed to coincide with the time of their maximum brain concentration. In addition, the levels of compounds achieved in the brain provide valuable data on the concentration of these compounds which should be used for in vitro studies to provide meaningful physiological results.

Experimental Section

Solvents and Chemicals—All solvents and chemicals used as starting materials for synthetic preparations were distilled prior to use.

Instrumentation—Mass spectra were recorded with a Finnigan model 3200 chemical ionization quadrupole spectrometer interfaced to the same manufacturer's Incos model 2300 Data System. The GC separations were achieved using U-shaped glass columns (1.5 m × 2 mm, i.d.) packed with 2% OV-17 on Gas Chrom Q (100–120 mesh, Applied Science, State College, PA). The GC injector temperature was maintained at 270 °C, and an initial oven temperature of 200 °C was programmed at 10 °C min^{-1} one minute after sample injection to a final temperature of 300 °C. Methane (flow = 20 mL min^{-1}) was used as the GC carrier gas and CI reagent gas (ion source pressure 120 Pa). The ion source temperature was maintained at a nominal 110 °C by a constant filament emission of 1 mA. Under these GC conditions, the following retention times were recorded: dihydrokawain, 5.6 min; kawain, 6.8 min; desmethoxyyangonin, 7.3 min; and yangonin, 9.3 min. The methane CI mass spectra of kava resin constituents have been reported elsewhere.[7]

Preparation of Deuterated Internal Standards—*[4-OC^2H_3]Kawain (2b)*—Diisopropylamine (1.41 mL, 10 mmol, Aldrich Chemical, Milwaukee, WI) was added to a flame-dried, two-necked, round-bottom flask (100 mL) equipped with a septum cap and magnetic stirrer, and the apparatus was continuously flushed with dry N_2 gas and cooled to −78 °C (dry ice). Tetrahydrofuran (15 mL, distilled from sodium) was injected into the flask, *n*-butyllithium (6 mL, 10 mmol, Aldrich) was added in a dropwise manner over a period of 30 min, and the reaction mixture was stirred for an additional 10 min when *trans*-cinnamaldehyde (0.63 mL, 5 mmol, Aldrich) was added. After 10 min, the bright yellow solution was warmed to 0 °C and the reaction was quenched by the addition of ammonium chloride solution (2 M; 5 mL). The reaction mixture was diluted with water (10 mL) and extracted with ether (2 × 20 mL) to yield a brown semisolid (1.23 g). Methane CI–GC–MS identified the major component as cinnamaldehyde and suggested the presence of **2a** whose methane CI–GC–MS spectrum had an ion at *m/z* 173, corresponding to the thermal loss of CO_2 from its nonobserved $[MH]^+$ ion. Dissolution of the crude product in ether and base extraction separated **2a** (219 mg), which had the desired $[MH]^+$ ion at *m/z* 217, after solid-probe methane CI–MS. Methylation with dimethyl sulfate yielded kawain (2), which had an identical GC retention time and methane CI–MS spectrum[7] to authentic material isolated from kava resin.

Methylation of **2a** (50 mg) with $[^2H_6]$dimethyl sulfate was accomplished in a Reacti-vial containing acetone (1.4 mL) and anhydrous K_2CO_3 (145 mg), and the reaction mixture was heated to 60 °C for 16 h. The desired [4-OC^2H_3]kawain (**2b**, 16 mg) on methane GC–MS had the required $[MH]^+$ ion at *m/z* 234 (98% $^2H_3$), with no contribution at *m/z* 231 corresponding to the $[MH]^+$ ion of kawain (2).

[3,5,7-(4-OC^2H_3)-$^2H_6$]Desmethoxyyangonin (3a)—Desmethoxyyangonin (3) was synthesized from benzaldehyde by a published procedure.[12] To desmethoxyyangonin (80 mg) and $[^2H_4]$methanol (2 mL), sodium (1 mg) was added and the contents were heated at 80 °C for 1.5 h. After removal of solvent (dry N_2 gas), the residue was dissolved in dichloromethane and washed with aqueous NaOH (0.4 M, 2 × 2.5 mL). The organic layer yielded a yellow solid (57 mg) shown by GC–MS to be **3a** with the following isotopic composition: 1% $^2H_8$, 2% $^2H_7$, 47% $^2H_6$, 39% $^2H_5$, 8% $^2H_4$, and 1% $^2H_3$. Five of the six incorporated deuterium atoms were located on the pyrone ring because the methane CI fragment ion at *m/z* 125 in the mass spectrum of **3** was predominantly located at *m/z* 130 in that of **3a**.

[$^2H_6$]Dihydrokawain (1a)—$[^2H_6]$Desmethoxyyangonin (**3a**, 30 mg) in ethyl acetate (15 mL) was hydrogenated over 10% Pd/C (25 mg) at room temperature for 5 h. The catalyst was filtered off and the reaction continued in ethyl acetate (15 mL) with fresh catalyst for 2 h at 60 °C. The resulting product (16.5 mg) had an identical methane CI–MS spectrum[7] and GC retention time to authentic dihydrokawain (**1**), and its isotopic composition was calculated from the $[MH]^+$ ion distribution to be 1% $^2H_7$, 50% $^2H_6$, 42% $^2H_5$, 6% $^2H_3$, and 1% $^2H_2$.

[12-OC^2H_3]Yangonin (6a)—4-Hydroxybenzaldehyde (2.2 g, Aldrich) was converted to 4-[OC^2H_3]benzaldehyde by methylation with $[^2H_6]$dimethyl sulfate (1.8 mL) in acetone (40 mL) containing anhydrous K_2CO_3 (5 g) during 18 h under reflux. The deuterated aldehyde was then converted to the desired 12-[OC^2H_3]yangonin (**6a**) by a published procedure.[12] The product had an isotopic purity of 98% $^2H_3$, with no detectable ion current at *m/z* 259 corresponding to the $[MH]^+$ of unlabeled yangonin (**6**).

Experimental Protocol—Male Balb/c mice (18–26 g) were used throughout at an ambient room temperature of 21–23 °C. Doses of 100 mg/kg were used for each pure kava constituent based on the observation[11] that these concentrations protected mice from maximal electroshock and were approximately one-half of the doses which caused loss of righting reflex in our animals. Similarly, crude kava resin was administered for synergistic studies at a dosage of 120 mg/kg. All compounds were given ip (0.1 mL/10 g body weight) after being solubilized in Cremefor (10%) and saline (90%).

Mice were sacrificed by decapitation 5, 15, 30, and 45 min after injection. Brains were removed, rinsed in cold distilled water, patted dry, transferred to clean dry tubes, and weighed.

Extraction of Brain Tissue—This procedure was based on that reported by Shih and Markey.[13] Brain tissue (290–330 mg) was homogenized (Dounce Hand Homogenizer) in ice-cold distilled water (5 mL). Then, EDTA (2%, 0.2 mL), perchloric acid (0.4 M, 2 mL), and a constant amount of the appropriate internal standard of the target compound was added. When working with mice which had received kava resin, constant amounts of all four deuterated internal standards were used. Samples were vortexed for 1 min, placed on a rotary mixer for 2 min, and centrifuged at 3000 rpm for 15 min. Supernatants were extracted with chloroform (2 × 3 mL), the organic layer was washed with NaOH solution (0.5 M, 2 mL), and centrifugation was used to separate the two phases. The organic layer was dried (Na_2SO_4), filtered, and blown to dryness under a stream of dry N_2 gas. Samples were reconstituted in ethyl acetate (20 μL), and 2-μL aliquots were used for GC–MS analysis.

Selected Ion Monitoring (SIM)—Analyses were conducted, using GC–MS and SIM, of the eight $[MH]^+$ ions of each of the four target compounds plus their four deuterated internal standards; these *m/z* values are recorded in Table I. Standard curves were determined using blank mice brains (no injection of kava substances) and known variable amounts of each compound, plus a constant quantity of deuterated internal standard, were added to each brain homogenate. Peak heights were used for the recording of ion current intensity values. Standard curves were linear (correlation coefficients varied between 0.999 and 0.972) in the range of the assay.

Quantitation of Dihydrokawain, Kawain, Desmethoxyyangonin, and Yangonin in Commercial Kava Resin—This was achieved using the procedure based on the above GC–MS and SIM technique for measuring these compounds in brain extracts. A solution of kava resin (5 mg/mL), prepared from commercial plant material from Vanuatu, was used in the animal studies at a dosage of 120 mg/kg. This dosage contained the concentrations of the four kava lactones

Table I—Ions Used for the Gas Chromatography—Mass Spectrometry Detection of Four Kava Constituents

Constituent	Ion[a]
Dihydrokawain	*m/z* 233; (*m/z* 239)
Kawain	*m/z* 231; (*m/z* 234)
Desmethoxyyangonin	*m/z* 229; (*m/z* 235)
Yangonin	*m/z* 259; (*m/z* 262)

[a] Values in parentheses refer to deuterated internal standards.

recorded in Table II. The four standard curves used in this assay were linear with correlation coefficients varying between 0.999 and 0.995.

Results and Discussion

Preparation of deuterated internal standards of the four kava constituents (see Scheme I) was achieved using an existing synthesis for yangonin[12] commencing with [4-OC^2H_3]benzaldehyde. Adaptation of a β-keto ester condensation reaction[14] yielded the kawain precursor (**2a**) which, on methylation with [$^2H_6$]dimethyl sulfate, afforded [4-OC^2H_3]kawain (**2b**). Desmethoxyyangonin (**3**) was conveniently labeled with deuterium by base-catalyzed exchange in perdeuteromethanol solution to give the [$^2H_6$]analogue (**3a**) which, on catalytic hydrogenation, afforded [$^2H_6$]dihydrokawain (**1a**).

Rasmussen et al.[15] attempted to measure by GC the octane–water partition coefficients of the major kava lactones and found that these compounds could not be detected in the aqueous phase. The rapid "brain" penetration of the lactones observed in the present study are consistent with the high lipid solubility of these compounds. Thus, maximal brain concentrations (ng/mg wet brain tissue) of the two 5,6-dihydro-α-pyrones, dihydrokawain (**1**; 64.7 ± 13.1) and kawain (**2**; 29.3 ± 0.8), were measured 5 min after their ip injection (100 mg/kg). Our results are in accord with the observation of Meyer[11] who reported that the peak effect of dihydrokawain and kawain in protecting mice from maximal electroshock seizure occurred after 10 min. Therefore, the brain concentrations correlate well with the centrally mediated pharmacological actions exhibited by these compounds.

The two α-pyrones, desmethoxyyangonin (**3**) and yangonin (**6**), did not exhibit the definitive trends shown by dihydrokawain and kawain. In these instances, the maximal brain concentrations were lower and followed a more diffuse profile (Figure 1) after their ip injection at a dose level of 100 mg/kg. Measured concentrations (ng/mg wet brain tissue) were 10.4 ± 1.5 for desmethoxyyangonin and 1.2 ± 0.3 for yangonin. Meyer[11] established that both the α-pyrones **3** and **6** possess only a weak central nervous system activity in mice when given ip.

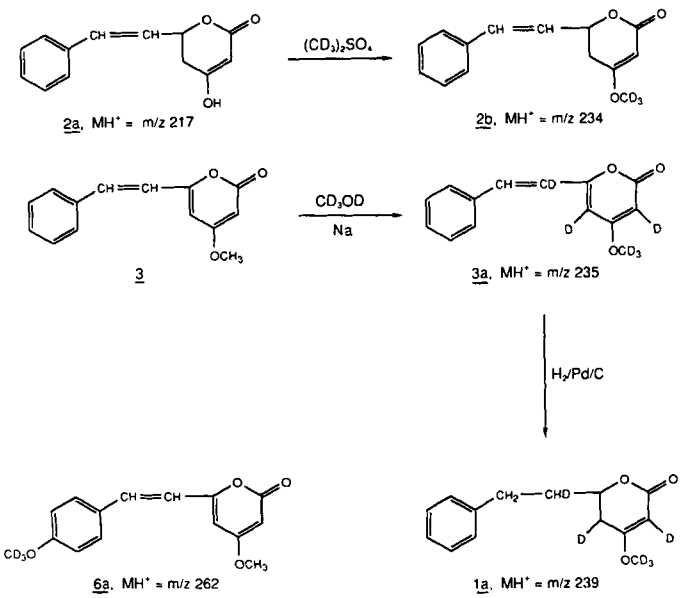
Having determined the brain levels of the four target kava constituents from their individual administration to mice, we investigated the proposed synergistic effect which has been previously reported[16] when crude kava resin was used in animal studies and compared with the effects of each individual compound. Kava resin isolated from commercial plant material from Vanuatu was first analyzed to determine the resin concentrations of the four components of interest, and these values are recorded in Table II. An ip dose of 120 mg/kg of kava resin resulted in the profile of brain concentrations for kawain, dihydrokawain, desmethoxyyangonin, and yangonin recorded in Figure 2. Thus, kawain administered at 100 mg/kg yielded (Figure 1) a brain concentration (ng/mg of wet brain tissue) of 29.3 ± 0.8, while from kava resin (120 mg/kg containing 44 mg/kg of **2**), a brain concentration of 27.8 ± 1.7 was attained. Clearly this supports the observation[16] that a synergistic effect operated in animal studies when kava resin was compared with its individual constituents acting alone.

A similar situation resulted with yangonin (**6**), where a single 100-mg/kg dose gave a brain concentration of 1.2 ± 0.3 ng/kg (Figure 1), and with resin (effectively 18 mg of **6**), which afforded a level of 4.6 ± 0.8 ng/mg (Figure 2); a relative increase of >20 times that measured after the administration of pure yangonin. Meyer also reported[11] that the activity of yangonin (administered ip) in preventing mice from maximal electroshock seizure was markedly increased when given in combination with other kava constituents. Thus, the synergism in pharmacological activity appears to be due to potentiation of penetration into the brain when the compounds are administered together rather than separately. The mechanism by which this may occur remains obscure. In view of their high lipid solubility, plasma binding may be

Table II—Commercial Vanuatu Kava Resin Analysis

Constituent	Concentration/120 mg of Resin, mg[a]
Dihydrokawain (**1**)	23
Kawain (**2**)	44
Desmethoxyyangonin (**3**)	16
Yangonin (**6**)	18

[a] Three other significant constituents of the resin, tetrahydroyangonin (**4**), dihydromethysticin (**7**), and methysticin (**8**), would account for the majority of the remainder (19 mg) of the resin dose.



Scheme I—*Chemical structures of the deuterated internal standards prepared for the GC–MS assay of dihydrokawain, kawain, desmethoxyyangonin, and yangonin (D = [^{2}H]). Protonated molecular ions [MH]$^+$ were recorded using methane CI–mass spectrometry.*

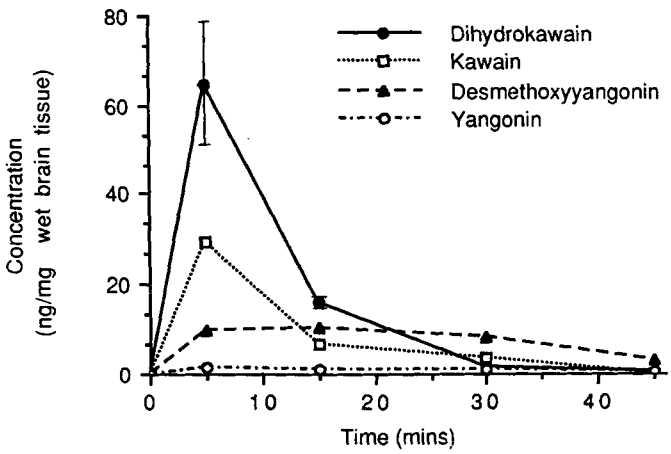


Figure 1—*Concentration of individual kava constituents in mouse brain at 5, 15, 30, and 45 min after a single ip dose (100 mg/kg). Each point represents the average of 6–9 analyses. Unless indicated to the contrary, error bars fell within the symbol area.*

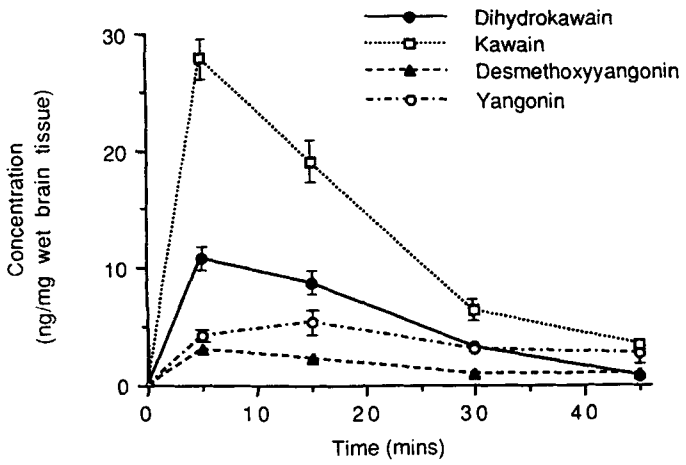


Figure 2—*Kava constituents measured in mouse brain after a single ip dose of kava resin (120 mg/kg). Each point represents the average of 6–9 analyses. Unless indicated to the contrary, error bars fell within the symbol area. Analysis of the kava resin is given in Table II.*

a significant factor, with competition for binding sites raising free plasma pyrone concentration. However, as plasma pyrone concentrations were not measured in the present experiments, it is not possible at this stage to judge whether absorption, distribution, or binding of pyrones is differentially affected when they are administered together rather than separately. Similarly, it has been reported that yangonin and desmethoxyyangonin were relatively ineffective when given orally. But, if given orally in combination with other kava constituents, a marked increase in overall potency occurred.[11] This implied that there is also a synergistic action in absorption of pyrones from the intestine when kava compounds are administered together rather than individually.

In contrast to the synergistic effects observed with kawain and yangonin following the administration of crude resin, the measured brain concentrations of desmethoxyyangonin and dihydrokawain remained (during the time course of the study) proportionally at the levels observed following their individual injection (see Figures 1 and 2).

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Acknowledgments

This work was supported by the National Health & Medical Research Council of Australia.