Research Article

Biomedical Chromatography

Received: 20 February 2008, Revised: 28 April 2008, Accepted: 29 April 2008 Published online 25 July 2008 in Wiley Interscience

(www.interscience.wiley.com) DOI 10.1002/bmc.1087

High-performance liquid chromatography assays for desmethoxyyangonin, methysticin, kavain and their microsomal metabolites

Shuang Fu,[a] Bruce N. Tattam,[a] Colin C. Duke[a] and Iqbal Ramzan[a]

ABSTRACT: Three novel, simple and reproducible high-performance liquid chromatography quantitative assays with UV detection were developed and validated for three major kavalactones—desmethoxyyangonin, methysticin and kavain—in rat liver microsomes using diazepam as an internal standard; liquid–liquid extraction was used for sample preparation and analysis was performed on a Shimadzu® 10A high-performance liquid chromatography system. The analysis was carried out in reversed-phase mode with a Luna® C_{18} column (150 × 2.00 mm, 3 μm) at 40°C. The limit of quantitation was 0.1 μg/mL using 0.25 mL of microsomal solution. The assays were linear over the range 0.1–10 μg/mL for desmethoxyyangonin, methysticin and kavain. Quality control samples exhibited good accuracy and precision with relative standard deviations lower than 15% and recoveries between 85 and 105%. The assays exhibited satisfactory performance with high sensitivity for quantifying desmethoxyyangonin, methysticin and kavain in rat liver microsomes and were successfully used to determine the three kavalactones and their microsomal metabolites. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: kava; desmethoxyyangonin; methysticin; kavain; high performance liquid chromatography; microsomal metabolites

Introduction

Kava-kava (*Piper methysticum* Forst.) has been used extensively in the Pacific as a drink for thousands of years. Roots are used to prepare a drink for social and ceremonial occasions, similar to coffee or tea in other cultures. The root is also used by local people for soothing the nerves and inducing relaxation and sleep (Singh, 1992). Various preparations of kava have also been marketed since 1980s in Europe and North America to manage mild anxiety and insomnia (Dentali, 1997). However, in March 2002, the United States Food and Drug Administration issued a consumer advisory that severe liver injury may be caused by dietary supplements containing kava (Center for Food Safety and Applied Nutrition, 2002). According to this advisory, in excess of 25 cases of liver injury associated with kava, including hepatitis, cirrhosis and liver failure, have been reported. The Centers for Disease Control and Prevention reviewed 10 such case reports (two in the United States, six in Germany and two in Switzerland) in which liver transplants were necessary following hepatic failure associated with kava-containing supplements (Centers for Disease Control and Prevention, 2002). Consequently kava has been banned in some Western countries.

The primary bioactive compounds in kava are kavalactones (KLTs). Eighteen KLTs have been identified; six major KLTs constitute ~96% of the lipid extract of dried roots and rhizomes. These are desmethoxyyangonin (DMY), yangonin (YGN), methysticin (MTS), dihydromethysticin (DHMTS), kavain (KA) and dihydrokavain (DHKA) (Ramzan and Tran, 2004). Figure 1 shows their chemical structures. KLTs have common structural features consisting of an aromatic ring, which is linked by a two carbon bridge to a methoxy-substituted unsaturated six-membered lactone ring. The KLTs differ with respect to the degree of unsaturation of the carbon bridge and/or lactone ring; and the presence or absence of substituents (hydroxy, methoxy, methylenedioxy substituents) on the aromatic ring.

Metabolism studies of KLTs are pre-requisite to solving kava's safety problems. Mathews *et al*. examined KLT inhibition of CYP450 enzymes and concluded that kava may cause adverse drug reactions via inhibition of drug metabolism (Mathews *et al*., 2002, 2005). Some KLT metabolites have been identified in several studies (Rasmussen *et al*., 1979; Johnson *et al*., 2003; Tarbah *et al*., 2003; Duffield *et al*., 1989; Zou *et al*., 2005). No *in vitro* enzyme kinetic data on the six major individual KLT are available.

A number of high-performance liquid chromatography (HPLC) methods with UV and/or MS detection have been described in the literature for separation and/or quantification of the six major KLTs simultaneously from whole kava extracts (Bilia *et al*., 2004). Tarbah *et al*. (2003) assayed kavain and its metabolites simultaneously by HPLC-DAD method. There are no validated assays available to quantify single KLT DMY and MTS individually.

In this study, the HPLC assays of KLTs were developed mainly for KLT enzyme kinetic studies to elucidate maximum rates of metabolite formation and characterize the principal cytochrome P450 enzymes involved and provide a plausible mechanistic explanation for hepatotoxicity arising from pharmacokinetic interactions. To move towards this objective, the metabolism of single KLTs has been individually examined, not the metabolism of a mixture of KLTs. Previous studies (Rasmussen *et al*., 1979; Duffield *et al*., 1989; Tarbah *et al*., 2003) reported that two of the major metabolites of KA and MTS, *p*-hydroxykavain (*p*-HKA) and *m*,*p*-dihydroxykavain (*m*,*p*-DHKA) were identical and also that DMY was one of the metabolites of KA. Simultaneous

* Correspondence to: I. Ramzan, Faculty of Pharmacy, The University of Sydney, NSW 2006, Australia. E-mail: dean@pharm.usyd.edu.au

[a] Faculty of Pharmacy, The University of Sydney, NSW 2006, Australia

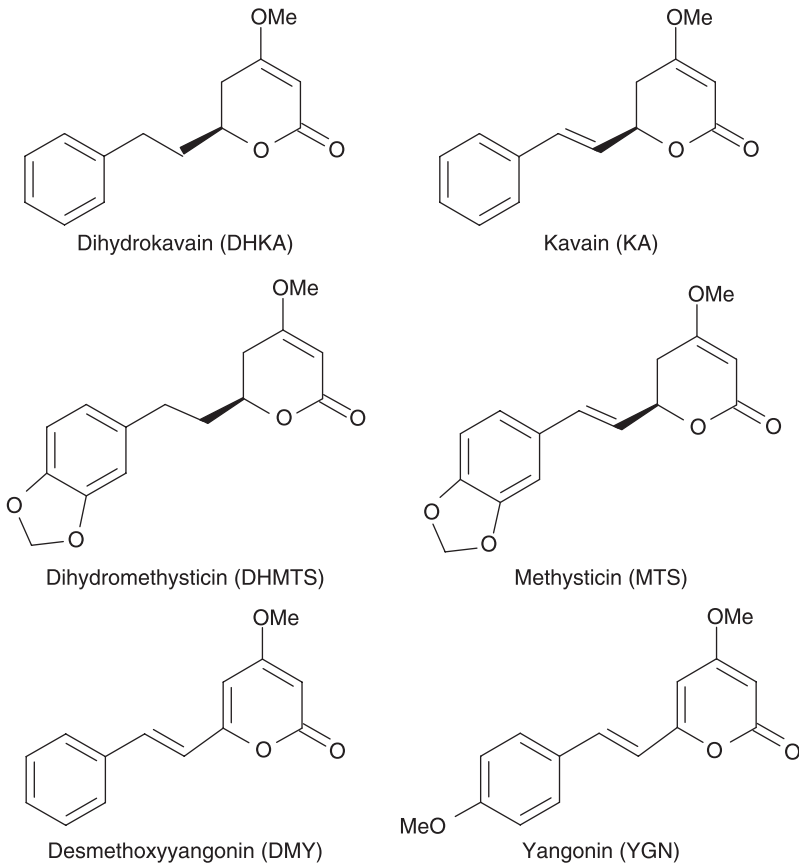

Figure 1. Chemical structure of six major kavalactones.

determination of metabolites from mixtures of KLTs is confounded by identical metabolites and metabolic interconversion between KLTs and, consequently, is not suitable for this study.

Effective assays are necessary to determine the concentration of individual KLTs and their metabolites in microsomal preparations to make it possible to study the kinetics and enzymes involved in their metabolism. Since there is no assay available for direct detection of the three major KLTs and their metabolites in rat hepatic microsomes, the aims of this study were to develop and validate simple, reproducible and effective quantitative HPLC assays to individually quantify major KLTs, namely DMY, MTS and KA and their possible metabolites simultaneously, in rat liver microsomes.

Experimental

Chemicals and Reagents

Desmethoxyyangonin and methysticin were isolated in this laboratory; their purity (>95%) and chemical structures were assessed by GC-MS/LC-MS, ^{13}C and ^{1}H NMR. Kavain (purity 95%) was purchased from ChromaDex, California, USA. HPLC-grade methanol was from LabScan Asia (Thailand) and HPLC-grade dichloromethane was from Mallinckrodt (USA). HPLC-grade 2-propanol (PrOH) and analytical-grade formic acid were from AJAX Chemicals (Australia). Diazepam (DZP), NADPH and all other reagents were analytical grade from Sigma-Aldrich (Australia).

Instrumentation

The HPLC system (Shimadzu® 10A) consisted of an SCL system controller, an SPD UV detector, an auto injector, C-R8A integrator and a Phenomenex® HPLC column heater. To obtain UV spectra for unknown peaks of interest, an LC-10ATVP Shimadzu pump, SIL-10ATVP auto injector, SPD-M10AVP diode array detector and SCL-10AVP system controller using the Shimadzu Class-VP chromatography data system (Shimadzu, Australia) were used.

Reversed-phase chromatography was performed with a Luna® (Phenomenex) C_{18} column (150 × 2.00 mm, 3 μm) at 40°C, using an isocratic mobile phase consisting of methanol and water (50:50, pH adjusted to 3.6 with formic acid). The mobile phase was filtered (0.2 μm) and degassed prior to use. The flow rate was 0.2 mL/min and injection volume was 50 μL. The UV detector was set at 205 nm for KA, 214 nm for DMY and 210 nm for MTS.

Liquid chromatography–mass spectrometry (LC-MS) and liquid chromatography–mass spectrometry–mass spectrometry(LC-MS-MS) analyses were performed on a Thermo Finnigan TSQ 7000 LC/MS/MS system (San Jose, CA, USA) in electrospray ionization mode (LC/ESI/MS) operated in the positive ion mode to analyze KLTs/metabolites of interest. Separation was achieved on an Agilent HP 1090 liquid chromatograph using the the same column and mobile phases at 40°C as described above, which facilitated identification of the compounds by yielding the same separation of the KLTs/metabolites over the 40 min acquisition time.

LC-MS-MS used argon as the collision gas at 2.0 mtorr and a corona current of 5 μA. The collision energy was set to 25 eV. The temperature of the heated capillary was set to 230°C. The precursor ion was selected for the MS/MS analysis and Q3 was scanned in full mass mode of 35–300 *m/z*.

Stock Solutions, Calibration and Quality Control Standards

Stock solutions of DMY, MTS, KA and DZP (internal standard) were prepared in methanol. Appropriate amounts of analytes were dissolved in methanol to a concentration of 1 mg/mL DMY, MTS, KA and DZP. Stock solutions were stored at –80°C. Working solutions of 0.1, 0.25, 0.5, 1, 2.5, 5 and 10 μg/mL of each KLT and quality control (QC) samples containing 0.2, 1.6 and 8 μg/mL were prepared by serial dilutions of the stock solution in methanol, and internal standard containing DZP 50 μg/mL. These solutions were protected from light and stored at –20°C, for no longer than 2 months.

Microsomal Incubation and Sample Extraction

For the microsomal incubations, an aliquot of 10 μL KLT working solutions of appropriate concentration was added to the reaction tube, and dried under a gentle nitrogen stream. Each incubation sample contained 0.125 mg (500 μg/mL) of rat liver microsomal protein and 0.1 M potassium phosphate buffer (pH 7.4) in a final volume of 0.25 mL. Samples were pre-incubated for 5 min at 37°C in a shaking (65 rpm) water bath; the reaction was initiated by the addition of 25 μL of 10 mM NADPH. Incubations were carried out for 30 min and then terminated by adding 5 mL of ice-cold 15% PrOH–dichloromethane mixture; after addition of 10 μL of DZP solution (50 μg/mL), the incubation mixture was then ready for extraction.

Calibration and quality control samples were prepared by combining 250 μL microsomal solution (after enzyme inactivation by 10 min of boiling), to 10 μL DMY or MTS or KA working solutions with 10 μL DZP, then addition of 5 mL of 15% PrOH in dichloromethane.

All samples were vigorously vortexed for 1 min and centrifuged at 3500 rpm for 20 min; the upper aqueous layer was discarded. The organic layer was evaporated under a gentle stream of nitrogen to dryness and the residues were reconstituted in 100 μL mobile phase; 50 μL was injected onto the HPLC.

Assay Validation

The three assays were validated according to FDA guidelines for bioanalytical method validation (Center for Drug Evaluation and Research, 2001), in terms of selectivity, linearity, limit of detection and quantitation, accuracy and precision, recovery, matrix effect and stability.

Selectivity. Selectivity of the assays was determined by comparing the chromatograms from blank microsomal samples with those of samples with three KLTs, metabolites of interest and DZP.

Calibration Curve Linearity. Linearity of the calibration curves were evaluated by linear regression of the peak area ratios of each KLT to DZP versus their respective microsomal concentrations. Triplicate assays were performed for each concentration. Five standard curves in one day and on five different days were used to assess assay performance. Assays were judged to be linear if the correlation coefficient (r^2) exceeded 0.995.

Limit of Detection and Quantitation. The limit of detection (LOD) for each KLT was the concentration at which the peak area ratio of analyte signal to noise was ≥3. The limit of quantitation (LOQ) was the lowest KLT concentration on the calibration curve that could be assayed with precision and accuracy less than 15%.

Accuracy and Precision. Intra- and inter-day accuracy and precision were determined at three QC microsomal concentrations. Accuracy was determined by comparing the assayed concentration to nominal concentrations using calibration curves. Precision was defined as the standard deviation of assayed concentrations to the mean of the assayed concentrations. Intra-day variability was assessed by analyzing QC samples five times within a day, and inter-day variability was determined by analyzing QC samples on five different days.

Recovery. Recoveries of KLTs at three QC concentrations and DZP were determined by comparing peak areas of spiked microsomal concentrations with those obtained by direct injections of the same amounts of analytes in mobile phase onto the HPLC without extraction.

Matrix Effect. Matrix effects were determined by comparing assayed QC concentrations prepared by dilution in methanol (C_s) to those obtained using blank microsomal solutions (C_m) spiked with corresponding amounts of KLTs.

Stability. Room temperature stability was assessed by storing three QC samples at room temperature (25 ± 2°C) for 4 h. Freeze–thaw stability was assessed following three freeze–thaw cycles using QC samples; three of these samples were thawed at room temperature and refrozen at –20°C over three cycles and assayed. Reconstituted QC samples were left in autosampler at room temperature for 24 h to assess sample stability in the autosampler. Three concentrations of QC stock solutions were prepared in methanol, stored at –80°C over 18 months and were assayed for KLT concentrations in comparison to a freshly prepared stock solution to assess long-term stability.

Rat Liver Microsome Preparation

Rat liver microsomes were prepared according to published method (Rettie *et al*., 1989) from 10 week-old-male Sprague–Dawley rats (Gore Hill, NSW, Australia) after approval from the Animal Ethics Committee of the University of Sydney (serial no. L24/8-2006/2/4445). Briefly, after rats were anaesthetized by pentobarbitone, their livers were perfused with saline and removed, and then homogenized in a hand-held Teflon-glass homogenizer in three volumes of buffer, containing 1 mM EDTA, 0.25 mM sucrose and 50 mM potassium phosphate (pH 7.4). Homogenized tissue was centrifuged at 20,000***g*** for 25 min at 4°C; the pellets were discarded and then the supernatant was centrifuged at 100,000***g*** for 70 min at 4°C. The microsomal pellet was resuspended in buffer and centrifuged at 100,000***g*** for 35 min at 4°C. The prepared microsomes were stored at –80°C in storage buffer containing 1 mM EDTA, 20% glycerol and 100 mM potassium phosphate (pH 7.4). Microsomal protein concentrations were determined by the method of Lowry *et al*. (1951) with bovine serum albumin as the standard.

KLT Enzyme Kinetics in Rat Liver Microsomes

Enzyme kinetics of the three KLTs were characterized *in vitro* by incubation of DMY, MTS and KA with rat liver microsomes at 37°C in a final volume of 250 μL. Time dependency of metabolism was determined using 10 μg/mL of each KLT with 0.125 mg microsomal protein for incubations lasting 5, 10, 20, 30, 45, 60, 90 and 120 min.

Data Analysis

KLT metabolites were identified by their apparent relative retention times to internal standard (IS) in HPLC and LC-MS chromatograms, UV spectra and mass from LC-MS and LC-MS-MS analysis. Data are expressed as the mean ± standard deviation of triplicate determinations. KLT concentration–time analyses were performed using the Scientist® software (Version 3.0, MicroMath Scientific Software, USA). Owing to the lack of authentic metabolite standards, the quantity of metabolites formed was expressed as the peak area ratio of each metabolite to the internal standard.

Results and Discussion

Materials and Equipment Resources

DMY, MTS and YGN were isolated from kava root in this laboratory. KA was purchased commercially; DHKA and DHMTS were not available commercially for this study. YGN was tested and found to be relatively unstable under the metabolic conditions, so only the major KLTs, namely DMY, MTS and KA, were studied further.

The purpose of the LC-MS analysis was to determine the identity of the metabolites (i.e. structure confirmation) as the HPLC assays were sensitive enough for this study. LC-MS analysis could have been used if higher sensitivity was required; however, HPLC alone is a more cost-effective tool where the analytes have no interferences from the background matrix.

Optimization of Chromatographic Conditions

The performance of the three HPLC assays was assessed in terms of linearity, LOQ and LOD, matrix effect, recovery, precision, accuracy, stability, peak shape and purity, and interference from endogenous substances in rat liver microsomes. Various HPLC chromatographic conditions were examined to obtain the best resolution of the three KLTs and their metabolites. The retention times of analytes were very sensitive to the ratio of methanol and water in mobile phase, the optimal separations of metabolites and KLT were achieved using methanol to water 50:50 at 40°C, and the flow rate was 0.2 mL/min. According to the UV spectra for DMY, MTS and KA and for the peaks assigned to KLT metabolites *p*-hydroxy-5,6-dehydrokavain (*p*-HDMY), *m*,*p*-dihydroxykavain (*m*,*p*-DHKA) and *p*-hydroxykavain (*p*-HKA), the maximal absorbances for DMY are 209 and 254 nm; for *p*-HDMY, 218 and 259 nm; for MTS, 207 and 305 nm; for *m*, *p*-DHKA, 213 nm; for KA, 204 and 246 nm; and for *p*-HKA, 207 and 261 nm. For optimum detection of KLT metabolites and parents, the UV wavelengths employed were 205 nm for KA, 214 nm for DMY and 210 nm MTS, at which the absorbances for metabolites and parents were maximal.

HPLC Chromatograms and UV Spectra

Representative HPLC chromatograms of DMY, MTS and KA in rat liver microsomal media are presented in Fig. 2. The retention times for DMY, MTS, KA and internal standard (DZP) were 31.5, 17.4, 18.5 and 37.0 min respectively; for major metabolite *p*-HDMY, *m*,*p*-DHKA and *p*-HKA retention times were 11.6, 4.4 and 6.2 min, respectively. No interfering peaks were noted at the retention times of analytes of interest. LC-MS analyses indicated that the tailing edge shoulder peak of MTS resulted from an isomeric impurity.

In UV spectra of DMY, MTS, KA and the peaks assigned to KLT metabolites *p*-HDMY, *m*,*p*-DHKA and *p*-HKA, the absorbance maxima for DMY were 209 and 254 nm; for *p*-HDMY, 218 and 259 nm; for MTS, 207 and 305 nm; for *m*,*p*-DHKA, 213 nm; for KA, 204 and 246 nm; and for *p*-HKA, 207 and 261 nm. The *p*-HDMY, KA and *p*-HKA absorbance maxima were identical to the values reported in the literature (Tarbah *et al*., 2003).

HPLC Validation Data

Selectivity. From Figure 2 it can be seen that there was no interference with any of the analyte peaks of interest. Retention times of MTS, KA, DMY and DZP were 16.5–17.5, 18–19.5, 31.5–33.5 and 36.0–37.0 min, respectively in the various HPLC assays. Metabolite retention times were 4.2–4.7 min for *m*,*p*-DHKA, 5.9–6.4 min for *p*-HKA and 10.8–11.8 min for *p*-HDMY.

Linearity and LOD/LOQ. Assays were found to be linear with the regression coefficients (r^2) for all calibration curves being greater than 0.9979. The LOQ was 0.1 μg/mL, with the relative standard deviation (RSD) less than 13.5%; and the LOD was 0.03 μg/mL for all three KLTs.

Accuracy and Precision. The intra- and inter-day accuracy and precision of the assays are presented in Table 1. Deviation from the true concentration was less than 15% for DMY and less than 9% for MTS and KA. The RSD in repeated analyses of the same samples was less than 10% for DMY, MTS and KA.

Recovery. The recoveries of DMY, KA and MTS extracted from rat liver microsomal media are presented in Table 2; and the recovery of DZP was 88–103%. Overall recovery was almost complete, with variability less than 15%.

Matrix Effect. Three KLTs were tested at three QC levels; the respective ratios of C_s to C_m were 103.74 ± 3.67, 108.39 ± 2.32 and 98.56 ± 6.46 % for DMY, MTS and KA, respectively. There were no significant matrix effects.

Stability. No significant losses (≤15%) of the three KLTs at low, medium and high QC levels were observed after storage at room temperature on the bench top over 4 h; reconstituted samples were stable in the autosampler for at least 24 h; samples were stable over three freeze–thaw cycles at –20°C up to 30 days. KLT solutions in methanol were stable up to 18 months at –80°C. Table 3 summarizes this stability data; the three KLT were stable over all steps of the assays.

Application of these Assays During Microsomal Metabolism

The three validated assays were successfully applied to determine DMY, MTS and KA concentrations in rat hepatic microsomal media for individual incubations of the KLTs. Figure 3 shows the concentration–time profiles of KLTs in rat liver microsomes

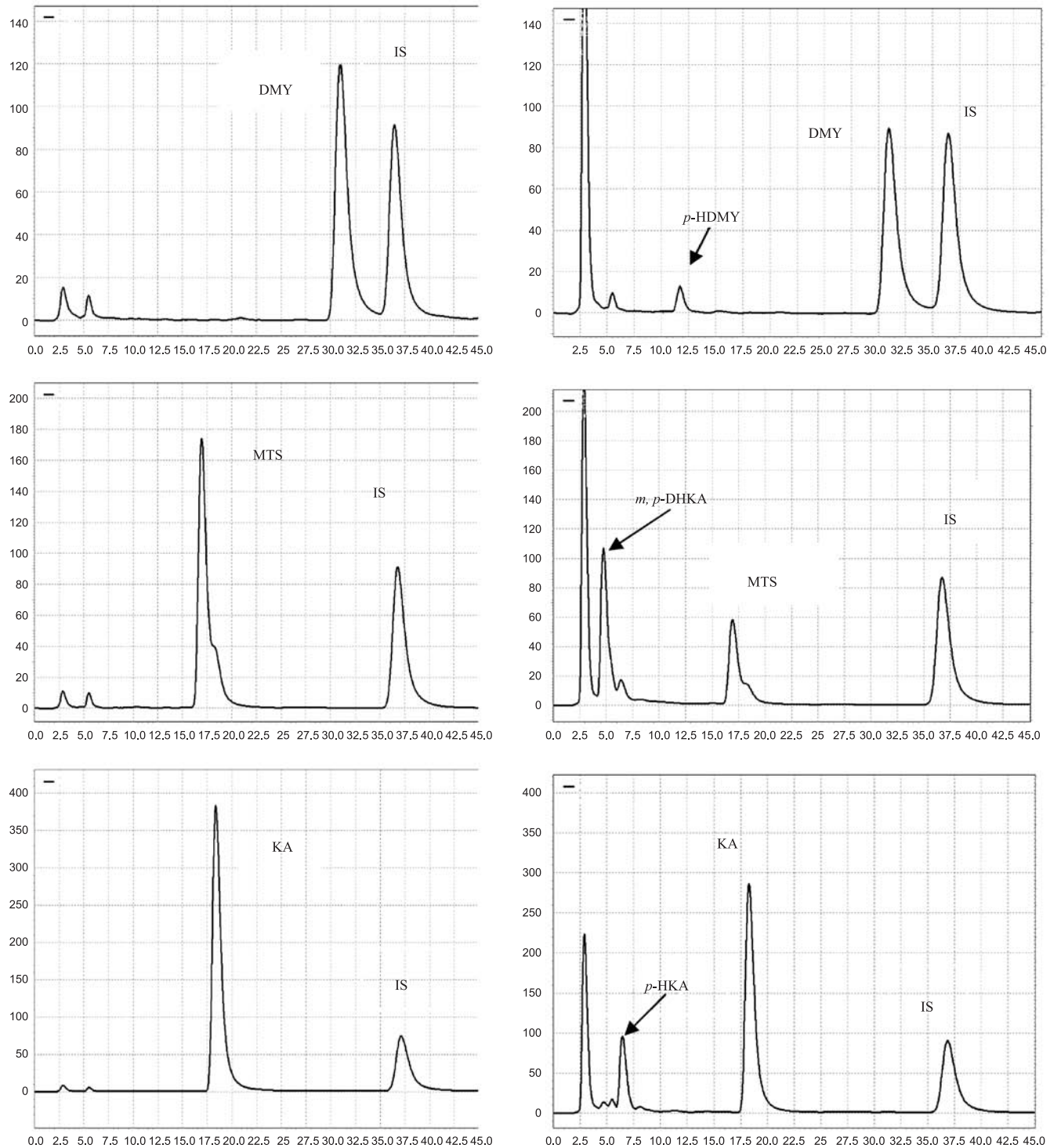

Figure 2. HPLC chromatograms of blank (no NADPH) microsomal media with individual KLT and IS (left panels), and DMY, MTS and KA after 30 min incubation in rat liver microsomes (right panels).

incubated for 120 min. The concentration of DMY and KA declined in a mono-exponential manner, and the concentration of MTS declined in a biphasic manner consistent with a rapid decline followed by a slow rate of decline, implying two first-order processes. Those were related to their metabolism and generation of major metabolites (Fig. 4).

According to literature (Rasmussen *et al*., 1979; Tarbah *et al*., 2003; Duffield *et al*., 1989) there are multiple metabolites of MTS and KA. In this study, four metabolites of KA and three metabolites of MTS and DMY were found and identified by LC-MS; however, except for the major metabolites HDMY of DMY, *m*,*p*-DHKA of MTS and HKA of KA, other metabolites were either at a concentration

Table 1. Intra- and inter-day accuracy and precision for HPLC assay of KLTs in rat liver microsomes

KLT	Nominal concentration (μg/mL)	Intra-day		Inter-day	
		Deviation (%)	RSD (%)	Deviation (%)	RSD (%)
DMY	0.2	7.53	7.16	3.77	4.75
	1.6	14.25	8.80	5.85	6.47
	8	5.85	5.51	5.37	7.64
MTS	0.2	−7.13	8.35	−8.3	8.25
	1.6	−6.48	1.11	1.44	4.87
	8	−6.14	4.48	−6.18	6.08
KA	0.2	−3.23	3.26	−8.63	6.79
	1.6	−2.71	2.92	−2.98	2.89
	8	−6.39	7.28	−3.96	2.38

Data are presented as mean ± SD ($n = 5$).

Table 2. Recovery of DMY, MTS and KA from rat liver microsomal media

KLT		KLT recovery (%)		
		QC_{low} (0.2 μg/mL)	QC_{medium} (1.6 μg/mL)	QC_{high} (8 μg/mL)
DMY	Intra-day	91.8 ± 4.8	89.5 ± 3.7	96.2 ± 8.9
	Inter-day	94.8 ± 3.1	90.6 ± 3.3	87.4 ± 2.3
MTS	Intra-day	95.4 ± 9.4	90.8 ± 5.8	96.2 ± 7.2
	Inter-day	95.9 ± 5.5	97.0 ± 5.6	90.1 ± 4.3
KA	Intra-day	99.9 ± 5	101.2 ± 2.9	97.8 ± 5.3
	Inter-day	92.3 ± 6.5	96.9 ± 5.3	96.1 ± 5.2

Data are presented as mean ± SD ($n = 5$).

Table 3. Stability of three KLTs during different phases of the assay

KLT	Nominal concentration (μg/mL)	Assay condition							
		Assayed concentration deviation (%)[a]							
		Room temperature, 4 h	Auto sampler, 24 h	Freeze–thaw day 1 (−20°C)	Freeze–thaw day 15 (−20°C)	Freeze–thaw day 30 (−20°C)	Stock solution, 6 months (−80°C)	Stock solution, 12 months (−80°C)	Stock solution, 18 months (−80°C)
DMY	0.2	−1.0	4.2	1.5	−6.4	−14.4	3.2	6.9	12.2
	1.6	−0.5	2.0	2.6	−11.7	−8.8	−5.7	−2.4	−7.4
	8	−1.5	1.7	1.2	7.0	−3.6	−4.0	−5.0	−9.5
MTS	0.2	−8.2	−0.3	2.4	−4.6	−13.5	−4.6	−7.4	−12.7
	1.6	−1.1	0.7	−0.8	−8.8	−11.0	−2.1	−5.2	−8.4
	8	−7.3	1.4	3.1	−4.0	−14.9	−6.2	−3.5	−8.6
KA	0.2	−3.0	1.3	−8.3	−14.2	−13.4	−2.6	−4.4	−10.3
	1.6	−0.5	3.8	4.1	−5.7	−10.1	−1.9	−2.8	−9.8
	8	−6.4	2.2	3.9	−5.2	−14.7	−3.5	−3.1	−9.1

[a] Assayed concentration deviation (%) was defined as [(nominal concentration − assayed concentration)/nominal concentration] × 100%.
All QC samples were analyzed in triplicate; mean values are reported.

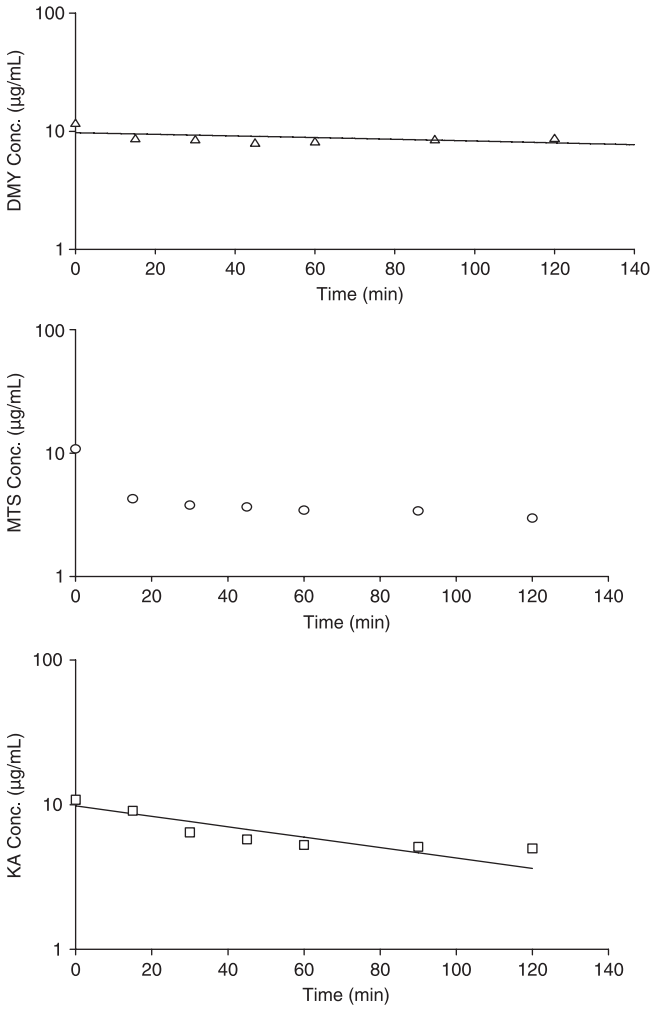

Figure 3. KLT concentration–time profiles during incubation in RLMs. For DMY and KA there is first-order mono-exponential metabolism (fitted lines); while for MTS there is an apparent bi-phasic decline in concentration, but due to limited number of data points a bi-exponential fit was not possible.

too low to detect, or rapidly degraded, so assays were not developed for the other metabolites.

Metabolite Structure Determination

Figures 5–7 are LC-MS spectra of three KLT major metabolites. Based on LC-MS-MS data, reference LC-MS-MS spectra (Tarbah *et al.*, 2003) and UV spectral evidence, the structure of the DMY major metabolite with a retention time of 11.61 min was determined to be *p*-hydroxy-5,6-dehydrokavain; the kavain major metabolite at retention time of 6.28 min was *p*-hydroxykavain. The MTS major metabolite at retention time of 4.37 min was determined to be *m,p*-dihydroxykavain from analysis of its LC-MS-MS spectrum and according to published literature (Rasmussen *et al.*, 1979). Table 4 summarizes the prominent MS-MS fragments of the major KLT metabolites. The postulated metabolite structures are shown in Fig. 8.

Tarbah *et al*. (2003) found *p*-hydroxy-5,6-dehydrokavain (*p*-HDMY) as a further metabolite from kavain's metabolite DMY. This study is the first reported identification of *p*-HDMY as the major metabolite of the kavalactone DMY in rat hepatic microsomes.

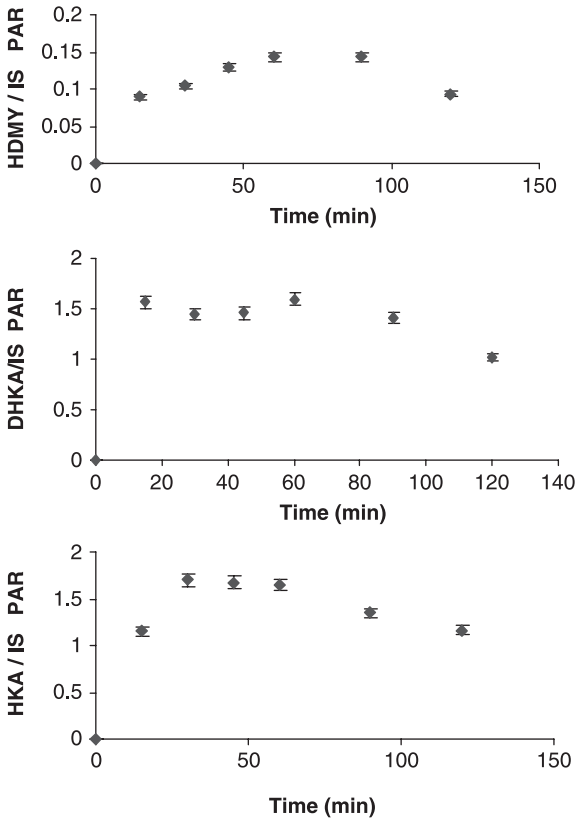

Figure 4. KLT metabolite (HDMY from DMY, DHKA from MTS and HKA from KA) formation as a function of time in RLMs. Metabolite formation is represented as metabolite to IS peak area ratio (PAR) as authentic metabolite standards were not available.

Table 4. Prominent MS/MS fragments from the protonated molecular ion of KLT metabolites

Metabolite	Retention time (min)	Molecular weight	Prominent fragments in decreasing order of *m/z* (relative intensity %)
DMY metabolite (*p*-HDMY)	11.61	244	217 (18), 185 (25), 157 (72), 147 (100), 139 (28), 128 (15), 119 (40), 91 (20)
MTS metabolite (*m,p*-DHKA)	4.37	262	213 (16), 199 (10),185 (20), 167 (10), 147 (100), 141(10), 99 (10)
KA metabolite (*p*-HKA)	6.28	246	197 (46), 169 (82),141 (70), 131 (100), 128 (20), 115 (15), 109 (32), 77 (20)

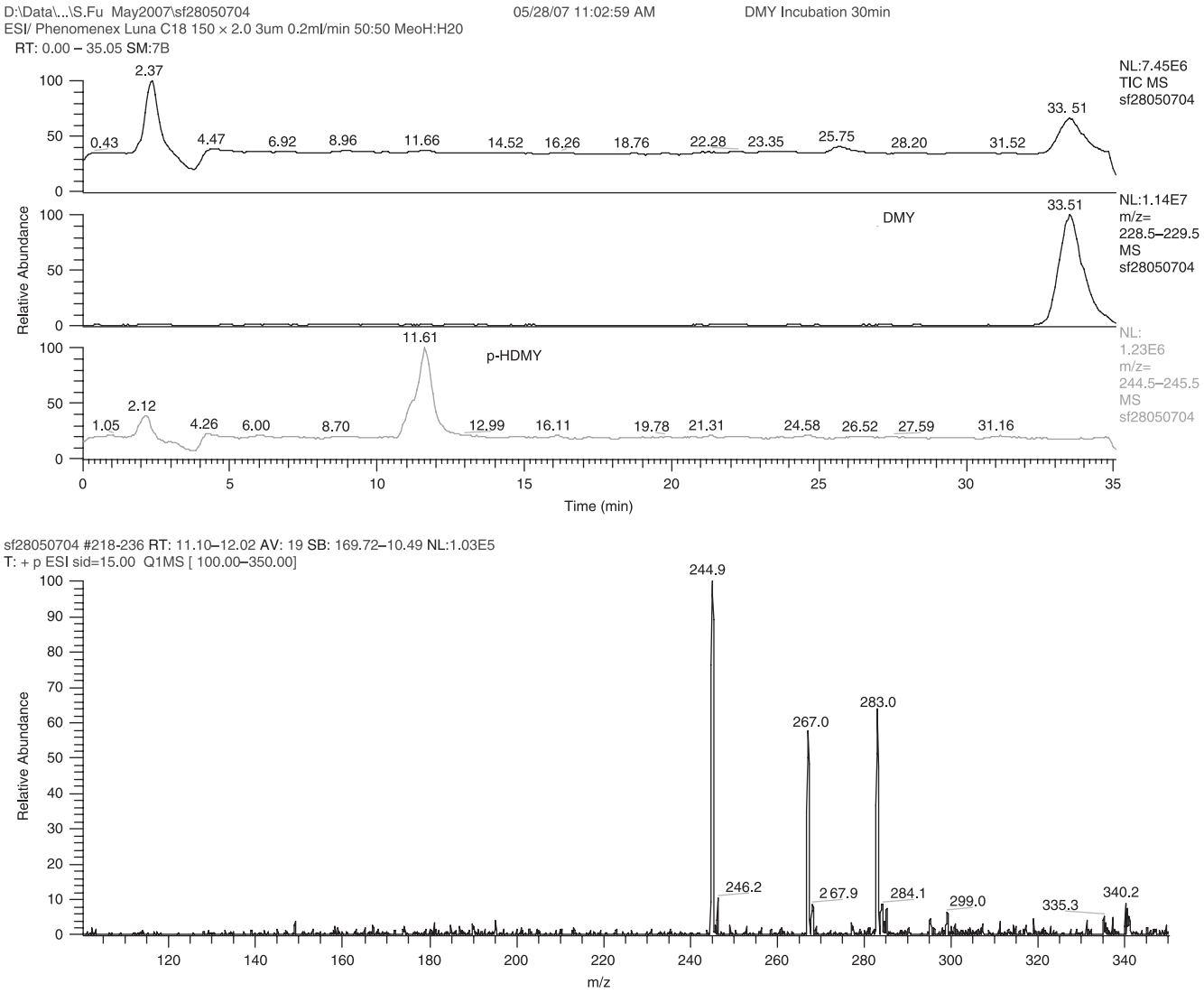

Figure 5. LC-MS spectra of DMY after 30 min incubation with RLMs. LC chromatogram (top) showing DMY retention time of 33.51 min and *p*-HDMY retention time of 11.61 min. MS spectrum (bottom) shows that the peak with retention time of 11.61 min corresponds to *m/z* of $[M + H]^+$ (244.9), $[M + Na]^+$(267.0) and $[M + K]^+$ (283.0).

Conclusions

Three simple, novel, sensitive, reproducible and selective HPLC assays for quantitative analysis of kavalactones desmethoxyangonin, methysticin and kavain in rat liver microsomes media were developed and fully validated. The assays were successfully used to study KLT metabolism in microsomal preparations. The results showed that microsomal metabolism of KLTs proceeded in either a mono- or bi-exponential manner. The HPLC assays also allowed quantification of the microsomal metabolites of these KLTs. In combination with LC-MS techniques, these metabolites could be identified and structures assigned. These assays should also be useful in quantifying the three kavalactones in pharmacokinetic studies in either humans or animals.

Acknowledgements

The authors appreciate the help and advice from Dr Rujee Duke with isolation of kavalactones and would like to thank Professor Andrew McLachlan and Mr Dennis Dwarte for assistance with data-fitting using Scientist®.

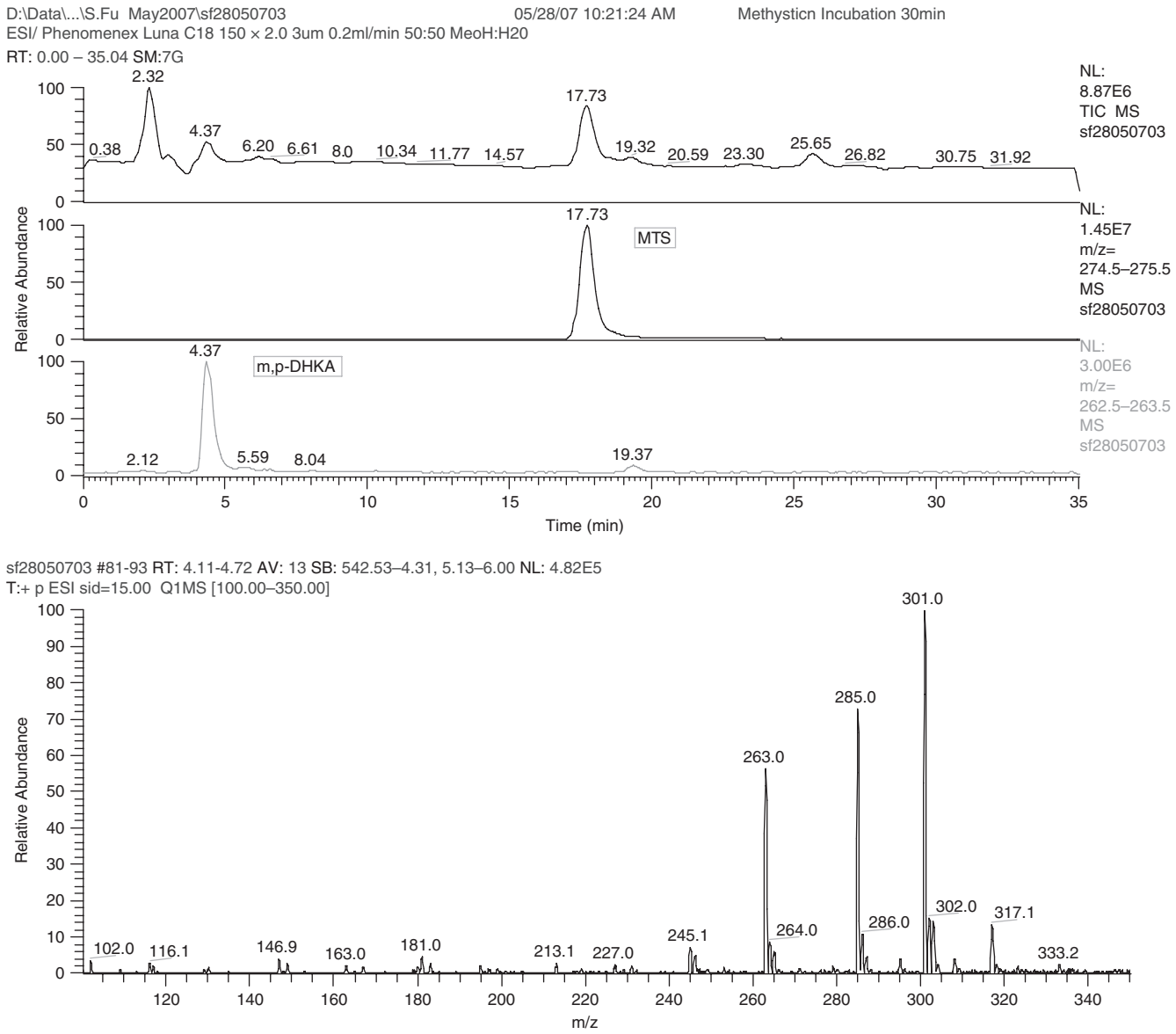

Figure 6. LC-MS spectra of MTS after 30 minutes of incubation with RLMs. LC chromatogram (top) showing MTS retention time of 17.73 min and m,p-DHKA retention time of 4.37 min. MS spectrum (bottom) shows that the peak with retention time of 4.37 min corresponds to *m/z* of $[M + H]^+$ (263.0), $[M + Na]^+$(285.0) and $[M + K]^+$(301.0).

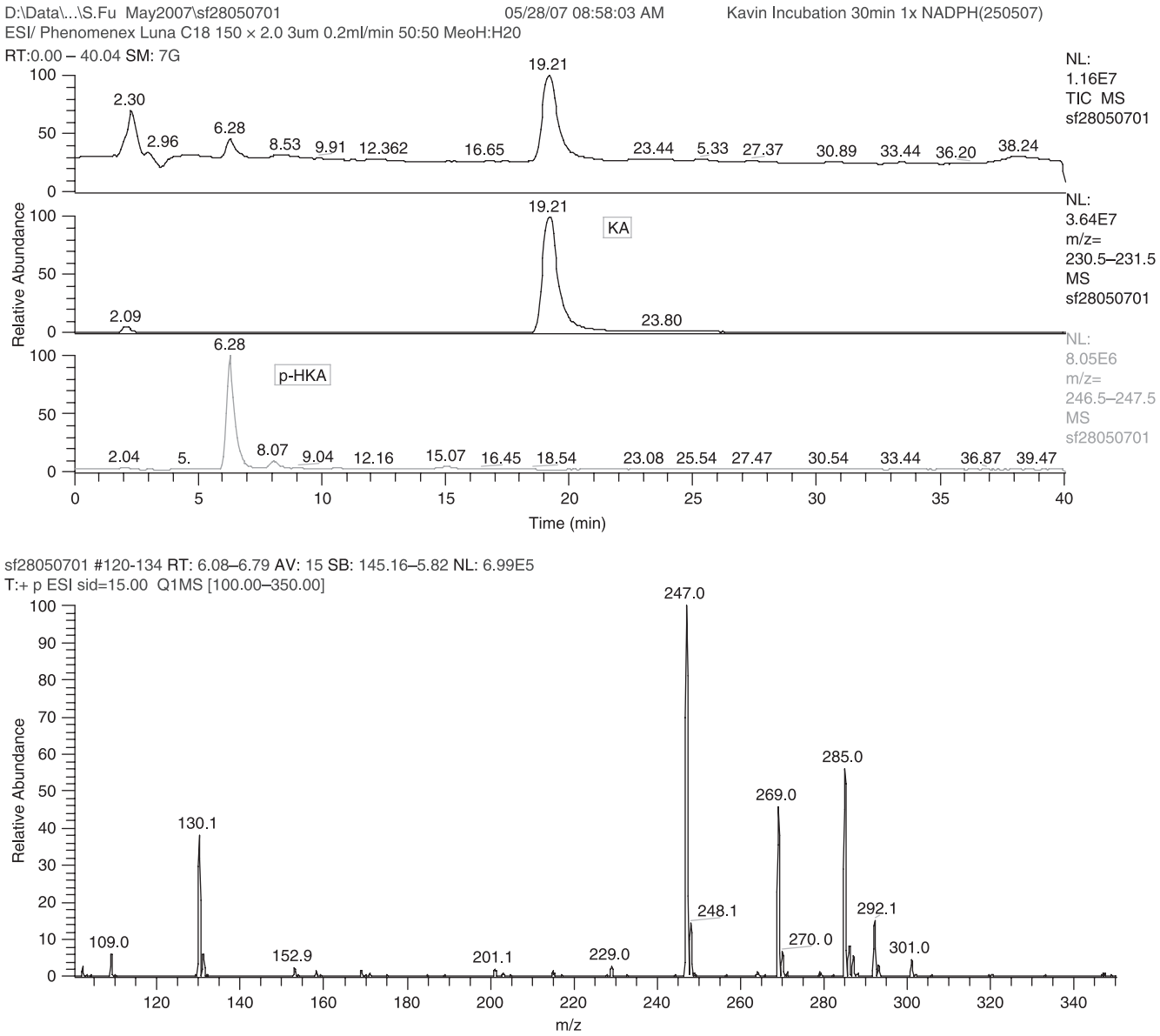

Figure 7. LC-MS spectra of KA after 30 minutes of incubation with RLMs. LC chromatogram (top) showing KA retention time of 19.21 min and *p*-HKA retention time of 6.28 min. The MS spectrum (bottom) shows that the peak with retention time of 6.28 min corresponds to *m/z* of $[M + H]^+$ (247.0), $[M + Na]^+$(269.0) and $[M + K]^+$(285.0).

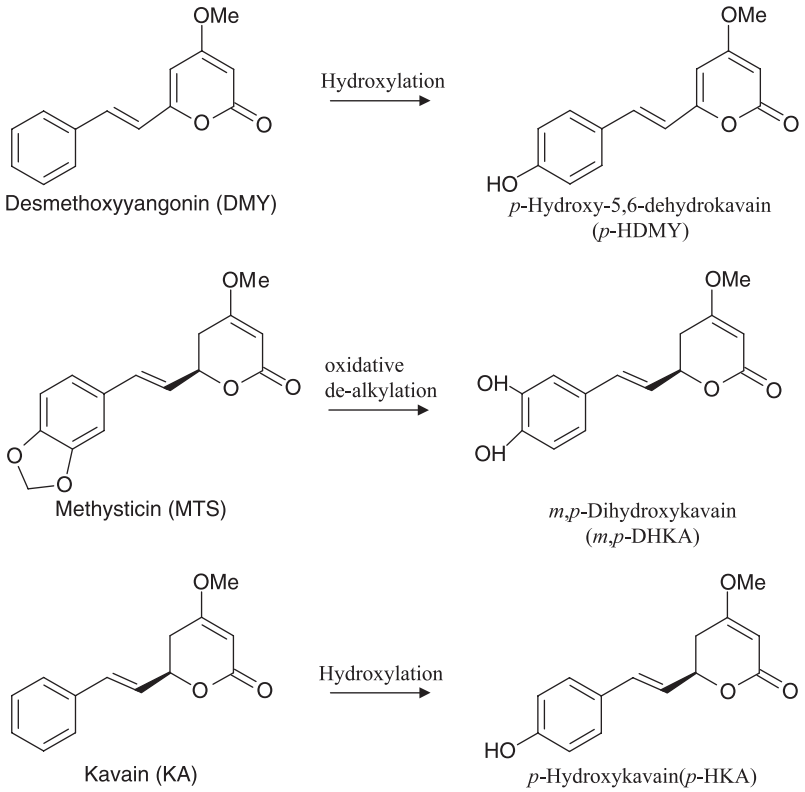

Figure 8. Postulated structures of the major metabolites of DMY (*p*-hydroxy-5,6-dehydrokavain, *p*-HDMY), MTS (*m*,*p*-dihydroxykavain, *m*,*p*-DHKA) and KA (*p*-hydroxykavain, *p*-HKA).

References

Bilia AR, Scalise L, Bergonzi MC and Vincieri FF. Analysis of kavalactones from *Piper methysticum* (kava-kava). *Journal of Chromatography B, Analytical Technologies in the Biomedical and Life Sciences 2004*; **812**: 203–214.

Center for Drug Evaluation and Research. *Guidance for Industry, Bioanalytical Method Validation*. US Department of Health and Human Services Food and Drug Administration, 2001.

Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, Kava-containing dietary supplements may be associated with liver injury, 25 March 2002). Available at: http://www.cfsan.fda.gov/~dms/addskava.html. Accessed 18 June 2005.

Centers for Disease Control and Prevention. Hepatic toxicity possible associated with kava-containing products—United States, Germany, and Switzerland, 1999–2002. *Journal of the American Medical Association*, 2002; **289**: 36–37.

Dentali SJ. *Herb Safety Review: Kava*. Herb Research Foundation: Boulder, Co, 1997; 6.

Duffield AM, Jamieson DD, Lidgard RO, Duffield PH and Bourne DJ. Identification of some human urinary metabolites of the intoxicating beverage kava. *Journal of Chromatography* 1989; **475**: 273–281.

Johnson BM, Qiu SX, Zhang S, Zhang F, Burdette JE, Yu L, Bolton JL and Van Breemen RB. Identification of novel electrophilic metabolites of *Piper methysticum* Forst. (Kava). *Chemical Research in Toxicology* 2003; **16**: 733–740.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* 1951; **193**: 265–275.

Mathews JM, Etheridge AS and Black SR. Inhibition of human cytochrome P450 activities by kava extract and kavalactones. *Drug Metabolism and Disposition* 2002; **30**: 1153–1157.

Mathews JM, Etheridge AS, Valentine JL, Black SR, Coleman DP, Patel P, So J and Burka LT. Pharmacokinetics and disposition of the kavalactone kawain: Interaction with kava extract and kavalactones *in vivo* and *in vitro*. *Drug Metabolism and Disposition* 2005; **33**: 1555–1563.

Ramzan I and Tran VH. *Kava from Ethnology to Pharmacology*, Singh YN (ed.). CRC Press: Boca Raton, FL, 2004; 76.

Rasmussen AK, Scheline RR, Solheim E and Haensel R. Metabolism of some kava pyrones in the rat. *Xenobiotica* 1979; **9**: 1–16.

Rettie AE, Eddy AC, Heimark LD, Gibaldi M and Trager WF. Characteristics of warfarin hydroxylation catalyzed by human liver microsomes. *Drug Metabolism and Disposition* 1989; **17**: 265–270.

Singh YN. Kava: an overview. *Journal of Ethnopharmacology* 1992; **37**, 13–45.

Tarbah F, Mahler H, Kardel B, Weinmann W, Hafner D and Daldrup T. Kinetics of kavain and its metabolites after oral application. *Journal of Chromatography, B: Analytical Technologies in the Biomedical and Life Sciences* 2003; **789**: 115–130.

Zou L, Harkey MR and Henderson GL. Synthesis, *in vitro* reactivity, and identification of 6-phenyl-3-hexen-2-one in human urine after kava-kava (*Piper methysticum*) ingestion. *Planta Medica* 2005; **71**: 142–146.

