MASS SPECTRAL CHARACTERISATION OF HEPATIC CELL METABOLITES OF D,L-KAVAIN USING HPLC AND GC/MS SYSTEMS

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ABSTRACT: The hepatic metabolism of D,L-kavain was studied in human urine and a human hepatic cell-line (Hep-G2). Samples were collected frequently, extracted and fractionated (HPLC). 23 fractions were collected and reanalysed (GC/MS) before and after derivatisation (methylation and silylation). Analysis of Hep-G2 extracts revealed 12 metabolites.

KEY WORDS: Kavain; Hep-G2 cells; GC/MS; Cell culture.

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INTRODUCTION

Kavain, a natural product from the roots of *Piper methysticum*, the kava plant, is a lactone related to α-pyrone, and is synthetically formed in two enantiomers [8].

An extract of the root and stem of this plant is called kava-kava, contains only the L-form and is used as a folk medicine as well as a ceremonial and social drink in the South Pacific.

The active principle of kava resin consists of a number of α-pyrones and reduced pyrones, which have a variety of pharmacological properties such as sedative, local anaesthetic, spasmolytic, smooth muscle relaxing, analgesic, antimycotic and antiedemic, and it is used as substitute therapy for benzodiazepine and alcohol as well as for a potentiation of barbiturate narcosis [2, 4, 9].

After oral administration of 200 mg D,L-kavain [6]: the initial resorption time in human subjects is about 15 min, the peak plasma concentration is reached in about 1.8 h, maximum plasma concentration is about 18 ng/ml, the distribution phase lasts about 3–5 h. Kavain concentration in blood decreases with a half-life of 9 h.

The aim of the present work was to study the metabolic pathway of kavain in human urine and in Hep-G2 cultures.

Hep-G2 cultures as the in vitro system, have the advantage such as:

– unlimited source;
– cheap and easy handling;
– reproducibility;

– persistent metabolic activities;
– retaining specialised liver functions and similarity to human liver [5].

17 kavain urinary metabolites are reported in literature [3, 6, 7].

MATERIALS AND METHODS

In vitro systems

– D,L-Kavain: Klinge Pharma GmbH München, Germany;
– Hep-G2 cells: ATCC, Rockville, Maryland, USA;
– cell culture medium: RPMI 1640-medium (10% fetal bovine serum) Penicillin and streptomycin 200 U/ml.

Urine samples were collected after 90 min and 120 min after intake of an oral dose of 800 mg kavain.

HPLC-system

– Perkin-Elmer series 3 LC-480;
– Column Kontrosorb 10 RP18;
– Perkin-Elmer LC-480 DAD.

GC/MS-system

– GC 5890/MSD 5970 with automatic liquid sampler HP 7673;
– column: HP-5MS;
– carrier gas: helium (70 kPa);
– split/purge off time 2 min;
– injector temperature 220°C; transfer – line temperature 280°C;
– temperature program: 60°C, 4°C/min, 200°C; 10°C/min, 300°C; 5 min.

Kavain metabolism studies using Hep-G2 cell cultures

– A single dose of 2–60 mg kavain in 200 ml cell culture media + DMSO (to improve kavain solubility and cell membrane permeability);
– cells were plated at $1x10^6$ cells/25 cm^2/5 ml culture media;
– cells were incubated at 37°C for periods of 3 h, 10 h, 30 h or 70 h;
– cell medium without cells was used for blank studies.

Hep-G2 cell cultures and urine fluid/fluid extraction method for HPLC and GC/MS-systems [10]

– 10 ml supernatant culture media or 5 ml urine were extracted in a separate funnel by fluid/fluid extraction procedure using a mixture of dichloromethane:ether (7:3, v/v);

– the organic phase was evaporated in vacuum;
– the neutral residue was dissolved in 50 µl methanol;
– 25 µl of the Hep-G2 cell culture extract were injected into HPLC;
– a linear gradient, programmed from 5% to 95% acetonitrile in water, was used;
– 23 fractions were collected, dried and resolved in methanol for GC/MS characterisation.

Derivatisation of kavain and the metabolites

– Methylation using iodomethane;
– silylation using N-methyl-N-trimethylsilyltrifluoroacetamide.

Isolation of kavain metabolites using Bond Elute C-18 columns

– Organic phase of fluid/fluid extraction were evaporated to dryness;
– residue was dissolved in borate buffer (pH 9);
– a Bond Elute C-18 column was preconditioned (methanol, water, borate buffer pH 9);
– residue solution was added, the column washed with 25% methanol/water;
– elution used methanol;
– methanolic extracts were reduced and analysed.

RESULTS AND DISCUSSION

Direct GC/MS analysis of the Hep-G2 extracts revealed five different metabolites of kavain M-I, M-IV, M-XI, M-XIII, M-XIV.

– The relative amount of metabolite M-IV significantly increased about 9-fold after enzymatic deglucuronisation;
– two derivatives of the metabolites M-X, M-IV could be only identified after methylation;
– six silylated derivatives of the metabolites M-I, M-III, M-V, M-XV, M-XVI, M-XVIII were detected, only five of them were identified;
– in addition, also the unchanged drug kavain was detected (Tables I–IV);

TABLE I. METABOLITES OF KAVAIN

R_1	X	R_2	Compound	Name	Hep-G2 cells	Human urine

H	CH = CH	H	P	Kavain	+++	+++
OH	CH = CH	H	M-I	Hydroxykavain	Traces	+
H	CH = CH	OH	M-II	5-Hydroxykavain	–	+
p–OH	CH = CH	H	M-III	p-Hydroxykavain	+	+
OH	CH_2–CH_2	H	M-IV	Hydroxydihydrokavain	+	+
p–OH	CH_2–CH_2	H	M-V	p-Hydroxydihydrokavain	+	–

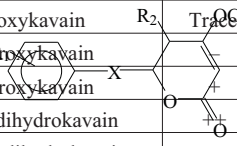

TABLE II. METABOLITES OF KAVAIN (CONTINUING)

R_1	X	Compound	Name	Hep-G2 cells	Human urine
H	CH = CH	M-VI	5,6-Dehydrokavain	–	+
OH	CH = CH	M-VII	Hydroxy-5,6-dehydrokavain	–	–
OH	CH_2–CH_2	M-VIII	7,8-Dihydro-5,6-dehydrokavain	–	–
p–OH	CH = CH	M-IX	p-Hydroxy-5,6-dehydrokavain	–	+

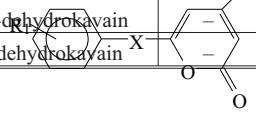

TABLE III. METABOLITES OF KAVAIN (CONTINUING)

R_1	R_2	Compound	Name	Hep-G2 Cells	Human Urine
H	CH=CH–C(OCH_3)=CH–CO_2H	M-X	Kava acid	+	+
H	CH(OH)–CH_2–$COCH_3$	M-XI	Cinnamylaceton	+	+
OH	CH(OH)–CH_2–$COCH_3$	M-XII	Hydroxycinnamyl-aceton	–	+
H	CO–CH_2–$COCH_3$	M-XIII	4-Oxycinnamyl aceton	+	–

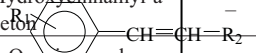

TABLE IV. METABOLITES OF KAVAIN (CONTINUING)

Chemical formula	Compound	Name	Hep-G2 Cells	Human urine
C_6H_5–CH_2–CH_2–CH=CH–$COCH_3$	M-XIV	6-Phenyl-3-hexen-2-one	+	+
HO–C_6H_4–COOH	M-XV	p-Hydroxybenzoic acid	+	–
Unidentified metabolite	M–XVI	323, 247, 115, 179, 143	+	–

Unknown metabolite	M–XVII	m/z 247, 105, 143, 135, 177, 134, 193, 205, 115, 260, 273	+	+
Unidentified metabolite	M–XVIII	m/z 260, 245, 115, 128, 127	Traces	+

– 12 of 17 previously published urinary metabolites could also be detected in Hep-G2 cells (Tables I–IV);
– the extraction of cell-culture media as well as human urine revealed a new metabolite (M-XVII) hitherto not described in the literature;
– the main metabolic pathway of kavain is hydroxylation of the aromatic ring. In addition, ring opening, hydroxylation as well as subsequent dehydration of the lactone ring and reduction of the 7,8-double bond are observed.

The metabolite M–X could only be detected in form of its methylated derivative.

– The five metabolites M-III, M-V, M-XV, M-XVI, M-XVIII could only be detected in form of their silylated derivatives;
– 8 kavain metabolites formed by Hep-G2 cells are identical to human urinary metabolites.

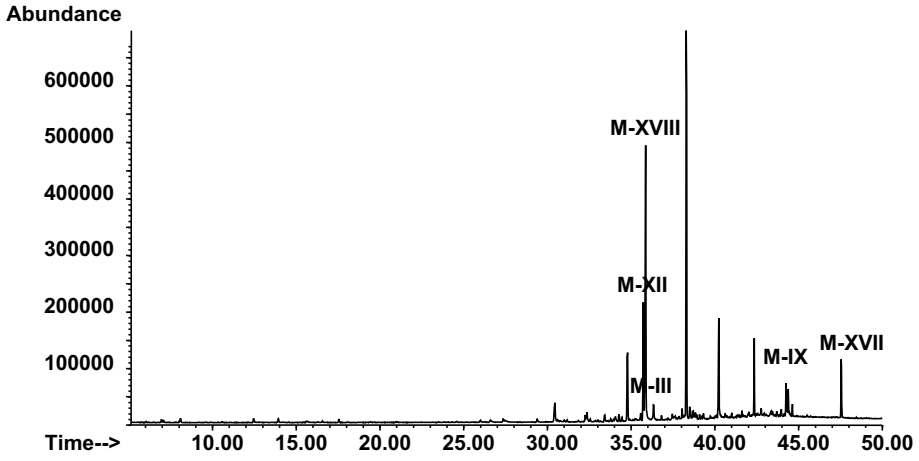

Fig. 1. Human urine extract (after silylation, GC/MS-chromatogram).

TABLE V. KAVAIN AND METABOLITES

Metabolite	Retention time [min]	Type of derivatisation	Prominent framents [m/z] in decreasing order of magnitude
Kavain	39.82	None	98, 68,91, 202, 230, 131, 128
M-I	49.10	None	107, 91, 121, 69, 133, 77

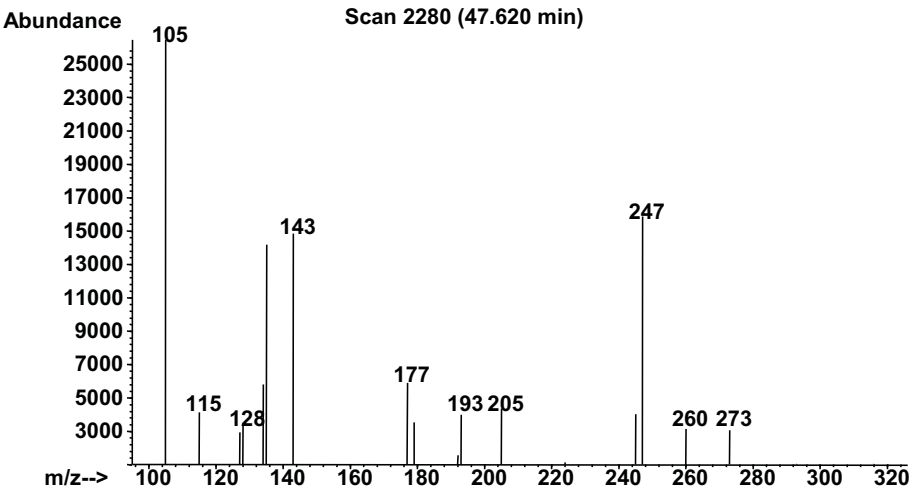

Fig. 2. Previously unpublished metabolite M-XVII detected in cell culture and urine extract (GC/MS-SIM).

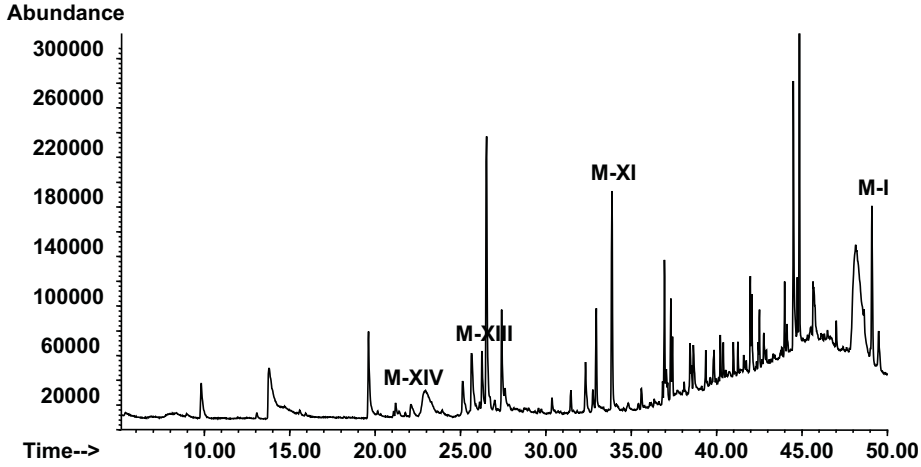

Fig. 3. Cell culture extract (underivatised, GC/MS-chromatogram).

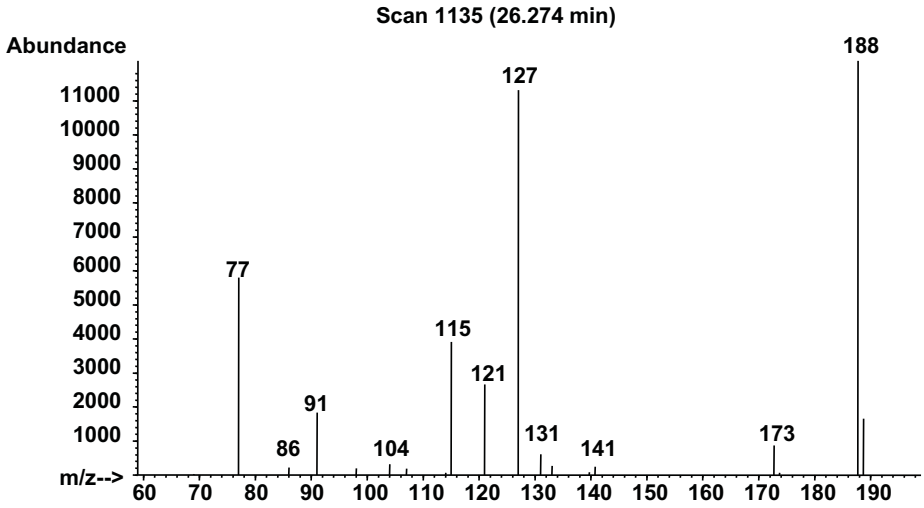

Fig. 4. Metabolite M-XIII (4-oxycinnamylaceton) detected only in cell culture extract (GC/MS-SIM)

M-II	Not detected	–	–
M-III	36.35	Silylation	179, 273, 115, 105, 127, 260
M-IV	47.22	Methylation	107, 133, 127, 77, 98, 189
M-V	19.81	Silylation	179, 205, 193, 105, 135, 128
M-VI	40.53	None	228, 69, 140, 77, 114, 131
M-VII	Not detected	–	–
M-VIII	Not detected	–	–
M-IX	44.38	Silylation	316, 245, 273, 115, 135
M-X	38.28	Methylation	185, 244, 170, 115, 151, 77
M-XI	33.39	None	104, 121, 77, 69, 133, 141
M-XII	35.73	Silylation	293, 179, 115, 143, 193
M-XIII	26.27	None	188, 127, 77, 115, 121, 91, 173
M-XIV	21.4	None	91, 131, 174, 115, 104,,77
M-XV	19.15	Silylation	224, 193, 177, 135, 115, 105
M-XVI	9.65	Silylation	323, 193, 179, 205, 293
M-XVII	47.55	Silylation	105, 274, 143, 135, 177, 134, 193, 205, 115, 260, 273
M–XVII	35.86	Silylation	260, 245, 115, 128, 127

CONCLUSION

The present study reveals that kavain is metabolised by Hep-G2 cells. One previously unpublished metabolite (structure still unknown), with a retention time 47.55 min, is present in both, human urine and Hep-G2 cell cultures.

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