Analytical studies on the kavain metabolism in human specimen and liver cell lines

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To my parents, my wife and my children

Contents

Abbreviations

Amu	Atomic mass unit
APCI	Atmospheric Pressure Chemical Ionization
AUC_inf	Area Under the Concentration-time-curve (extrapolated to infinity)
C last	Concentration at T last
C max	Maximal Concentration
CI	Chemical Ionization
CID	Collision Induced Dissociation
Cl_F	Apparent Clearance
Cl renal	Renal Clearance
CPS	Cycle Per Second
CV	Coefficient of Variation
DAD	Diode Array Detector
DMSO	Dimethylsulfoxide
E. coli	Escherichia coli
EI	Electron Impact
ESI	Electron Spray Ionization
ESI/CID	Electron Spray Ionization / Collision Induced Dissociation
GC/FID	Gas Chromatography Flam Ionized Detector
GC/MS	Gas Chromatography Mass Spectrometry
GC/PND	Gas Chromatography Phosphorous-Nitrogen Detector
Hep-G2	Human hepatocellular carcinoma
H. pomatia	Helix pomatia
HPLC	High Performance Liquid Chromatography
LC	Liquid Chromatography
LOD	Limit of Detection
m/z	Mass to charge ratio
MRT	Mean Residence Time
MS	Mass Spectrometry
MS/MS	Tandem- Mass Spectrometry
MSTFA	N-methyl-N-trimethylsilyltrifluoro acetamide
OR	Orifice Voltage
Q1-scan	Single quadrupole mode
rpm	Revolution per minute
RT	retention Time
SD	Standard Deviation
SIM	Selected Ion Monitoring
SPE	Solid Phase Extraction
T lag	Lag time before onset of kinetics
T last	Time of last data point
T max	Time of maximal concentration
T_{50}	Half-life during terminal slope
TMAH	Tetramethyl ammonium hydroxide
UDP	Uridine phosphate
UV	Ultra Violet
Vz_F	Apparent volume of distribution
λ_z	Rate constant of terminal-slope

1 Introduction

1.1 Kava-Kava History

Kavain is one of the main active components of the rhizomes and roots of Piper methysticum G. Forst. (meaning intoxicating or narcotic pepper, family Piperaceae) [Kaul and Joshi 2001]. Common names are kava-kava, awa, hereafter referred to as kava. It is a perennial shrub resembling bamboo [Kaul and Joshi 2001, Czygan and Hiller 2001, Hölzl et al. 1994, Hänsel 1996 and Singh 1992].

Piper methysticum belongs to the most important harvest in its area of circulation e. g. the islands of the South Pacific such as Oceania and is cultivated with considerable effort. The rhizomes and roots of the kava plant are being pounded or chewed and doused with coconut milk or cold water.

The origins of kava usage in Oceania are unknown. Kava drinking itself is much older than any written history of this part of the world. Kava is an old age drink that was the beverage of choice for the royal families of the South Pacific. Believed to originate from Melanesia, kava grows abundantly in the sun-drenched islands of Polynesia. The kava plant is not spread by seed, but by the cutting of cultivates which are transported and replanted by humans.

Kava has been used in these cultures for a variety of disorders, some based on real effects and others on mere hearsay. The past two decades of investigation on kavalactones, which are claimed to be the active principles of the herb, have revealed anticonvulsive, analgesic, anxiolytic and centrally acting muscle relaxant activities [Kaul and Joshi 2001]. Kava, predominantly in form of ethanolic extracts, is also being prepared as pharmaceutical products. While extracts of Piper methysticum contain only the l-form of kavain, the synthetic preparations of kavain as pharmaceutical product are distributed as two enantiomers [Sauer and Hänsel 1967].

1.2 Recreational use of kava beverages

Kava has recently become a drug of abuse amongst some of the aboriginal communities in Australia where kava is imported mainly from Fiji and Vanuatu.

Aqueous extraction of the commercially dried plant material is used for the preparation of kava beverage [Barguil et al. 2002 and Cabalion et al. 2003]. It is abused due to its sedative and relaxation properties.

The main requirements for beverage preparation are the kava stock, bowl, cup, strainer and water. Depending on the nature of the occasion, kava would be in the form of fine roots, or rhizomes and stems, which are then reduced to fine particles. Alternatively commercially prepared powder may be used. Chewing is the other method for the preparation of kava. Chewing was normally done by young men or women. Due to kava local anaesthetics effects in mouth, the chewers had to have good, strong teeth and jaws, a clean mouth, and they had to be free of ailments e. g. coughs, colds and sores [Singh 1992].

Nowadays, for instance in New Caledonian kava-bars, kava is prepared by crushing the dry roots of Piper methysticum in tap water in a basin. It is then filtered with a tissue or a sock before drinking [Barguil et al. 2002].

Acute kava misuse leads to reversible anaesthesia of the mouth and skin, euphoria, sedation, muscle weakness, ataxia and eventually intoxication [Alexander 1985, Alexander et al. 1987, Cawte 1985, Gajdusek 1979, Frater 1976].

1.3 Medicinal use of kava

In many parts of the Pacific, it was generally thought that kava judiciously had a beneficial health effect. Kava was used for many medicinal purposes e. g. to soothe the nerves, to induce relaxation and sleep or to counteract fatigue. Kava drinkers believe kava to restore strength, to soothe stomach pains, and to cure ailments such as boils. In addition to drinking the pounded root, some people use kava leaves. Fumigation with the leaves is believed to treat general illnesses. Macerated kava as well as external application of the masticated kava stump are other methods of cure, although drinking it in the traditional way is the most popular method of cure.

Kava was extensively used in Germany before World War I in the manufacture of certain drugs and medicines [Singh 1992]. Kava was used in Europe before World War I as a treatment for gonorrhoea, cystitis and gout. Kava was also used as hypnotic, sedative, local anaesthetic, spasmolytic, smooth muscle relaxant, analgesic and antimycotic [Singh 1992, Kinzler et al. 1991].

The German Commission E, a group of physicians, pharmacists, pharmacologists and toxicologists, as well as representatives of the pharmaceutical industries and directive persons, established in 1978 and responsible for evaluating the safety and efficacy of herbal medicine reviewed the data on kava and in 1990 approved its use for conditions such as nervous anxiety, stress and restlessness [Schulz and Hänsel 1996, Scherer 1998, Schenk and Ploss 2000, Pittler and Ernst 2000, Blumenthal et al. 1998, Czygan and Hiller 2002, Kaul and Joshi 2001]. Kava liver toxicity was previously studied especially in a coastal Aboriginal community [Mathews et al. 1988]. The conclusion of his study that kava affects the liver was based on the markedly elevated plasma levels of γ-glutamyltransferase observed in kava users. Recently, several reports about hepatitis associated with kava consume as well as several cases of fulminant liver failure and toxic liver injury correlated to the medication with kava-kava [Stoller 2000, Humbertson et al. 2001, Russmann and Helbling 2001, Brauer et al. 2001, Saß et al. 2001, Escher et al. 2001, Strahl et al. 2001, Kraft et al. 2001, Barguil et al. 2002, Schmidt et al. 2002, Bilia et al. 2002, Schulze et al. 2003, Stickel et al. 2003, Teschke 2003, Humberston et al. 2003, Moulds and Malani 2003, Cairney et al. 2003, Russmann et al. 2003] heated the discussion about the use of kavain and kava extracts as a phytopharmaceutical substance in human medicine. While the banning of kavain and kava-extract in the German market through the Bundesinstitut für Arzneimittel und Medizinprodukte (BfArM) is applied, German scientists did not yet join in the banning debate [Loew and Gaus 2002, Schmidt and Nahrstedt 2002] also authorities of other countries did not yet join in the banning debate too, mainly because a strict proof for kava-extracts or particularly kavain as the toxic principle behind the liver failure still seems to be uncertain. For example Kraft et al. [2001] made the correlation between the liver failure and the consumption of kava-kava only by an exclusion of other causes.

1.4 Chemistry of kava-kava rhizomes

The main active components of kava roots are kavalactones or kavapyrones which are lipid soluble materials. The chemical analysis of the kava roots shows that the fresh material on average contains 80 % water. The dried root consists of about 43 % starch, 20 % fibers, 12 % water, 3.2 % carbohydrates, 3.6 % proteins, 3.2 % minerals and 15 % kavalactones. The lactone content varies between 3 to 20 % of the dry

roots, depending on the age of the plant. Kava resin containing the biological active lactones can be extracted with organic solvents [Kaul and Joshi 2001, Czygan and Hiller 2001, Hölzl et al. 1994, Hänsel 1996 and Singh 1992].

The determination of the relative amounts of the various compounds present in the intact plant is difficult, as different separation procedures lead to different analyses. A separation into major and minor kavapyrones components has been made as follows: enolidpyrone type (kavain and methysticine and dienolidpyrone type yangonin) and some other miscellaneous compounds [Hänsel 1996].

1.5 Compounds isolated from the kava roots and rhizomes

1.5.1 Kavalactones or kavapyrones

Eighteen lactones have been isolated from the kava rootstock [Hänsel 1968, Duve 1981 and He et al. 1997], ten of which e.g. kavain, 5,6-dehydrokavain, 7,8-dihydrokavain, methysticin, 5,6-dehydromethysticin, 7,8-dihydromethysticin, yangonin, 5,6-dihydroyangonin, 7,8-dihydroyangonin and tetrahydroyangonin have been defined as major components of kava. These kava compounds are substituted α-pyrones (Figure 1).

The so called marker components in kava extract are the following six kava lactones: yangonin, methysticin, dihydromethysticin, kavain, 7,8-dihydrokavain and 5,6-dehydrokavain. They are used to assess the quality of the pharmaceutical preparation.

The six essential kava lactones are variable in their quantities depending on the territorial origin of the plant. The total amount of the kava lactones in the raw material ranges between 5 to 8 % [Kaul and Joshi 2001, Czygan and Hiller 2001, Hölzl et al. 1994 and Hänsel 1996]. The main substances are the styrylpyrones kavain and methysticin, which ranged between 1 to 2 %. The amount of 7,8-hydrogenated α-pyrones such as 7,8-dihydrokavain and 7,8-dihydromethysticin is lower with 0.5 to 1 % [Coclers et al. 1969, Hänsel and Laraz 1985].

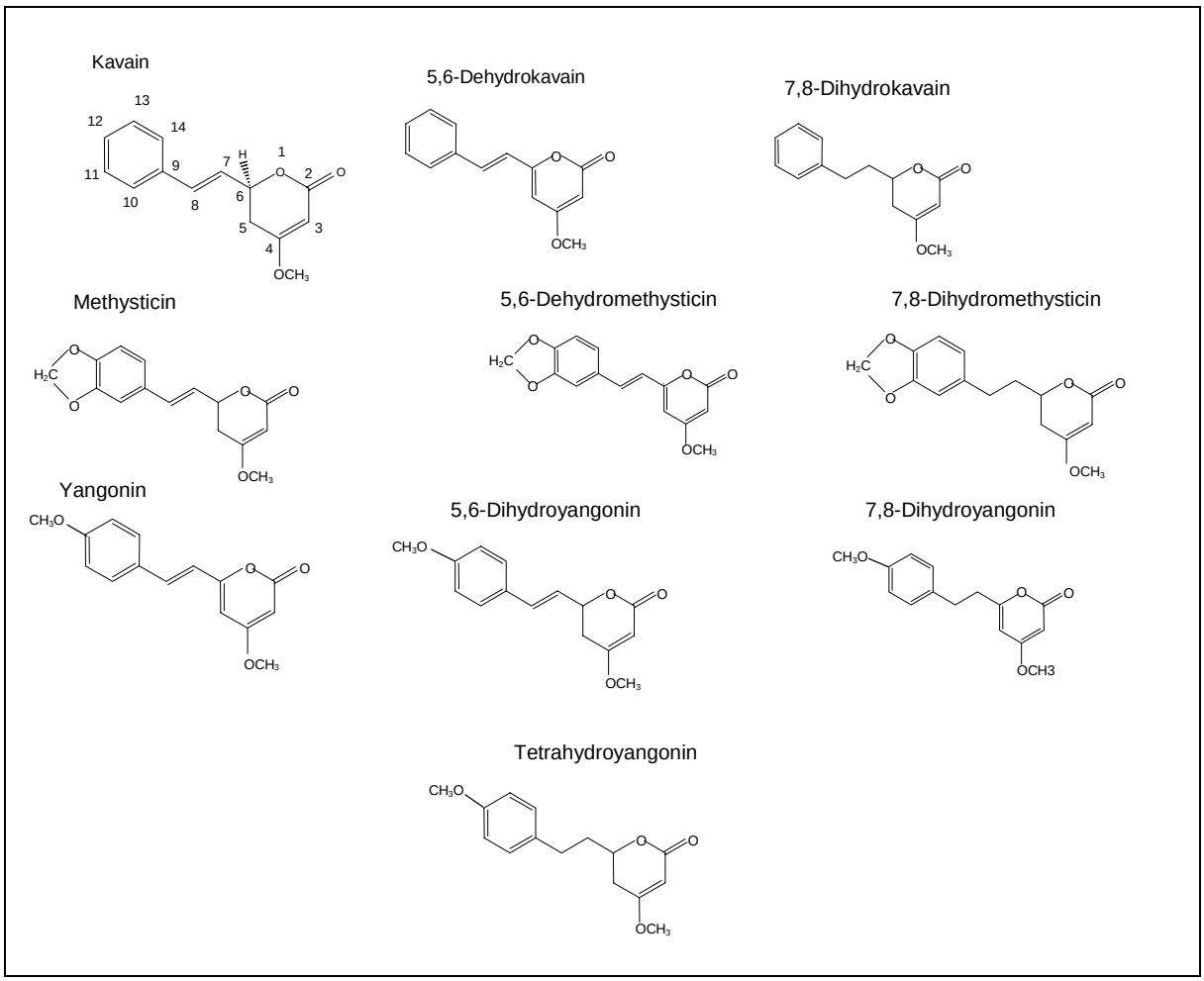


Figure 1: Chemical structures and names of the major components of kava

The total amount of the kavalactones or kavapyrones in kava roots or rhizomes is 3 to 5 % (according to DAC, the rhizomes should contain at least 3.5 % kavain). Therefrom are 1 to 2 % kavain, 0.6 to 1 % dihydrokavain, 1.2 to 2 % methysticin, 0.5 to 0.8 % dihydromethysticin and 0.9 to 1.7 % yangonin and desmethoxy-yangonin [Gracza and Ruff 1980, Hänsel and Woelk 1995, Hölz et al. 1994 and Hänsel and Kammerer 1996].

Recently the following distribution of the major kava lactones were found in kava extracts: total kava lactones 29.93 %, yangonin 4.30 %, methysticin 4.06 %, dihydromethysticin 6.35 %, kavain 6.08 %, dihydrokavain 7.29 % and desmethoxyyangonin 1.85 % [YMC Co. Ltd. Waters Millennium[32] and INAnetwork.com, Method for kava kava 2001].

Eight other compounds are minor compounds in the root stock (Figure 2): 5α-hydroxykavain, 11-methoxy-yangonin, 11-hydroxy-yangonin,

12-methoxy-11-hydroxy-5,6-dehydrokavain and 13-methoxy-yangonin.

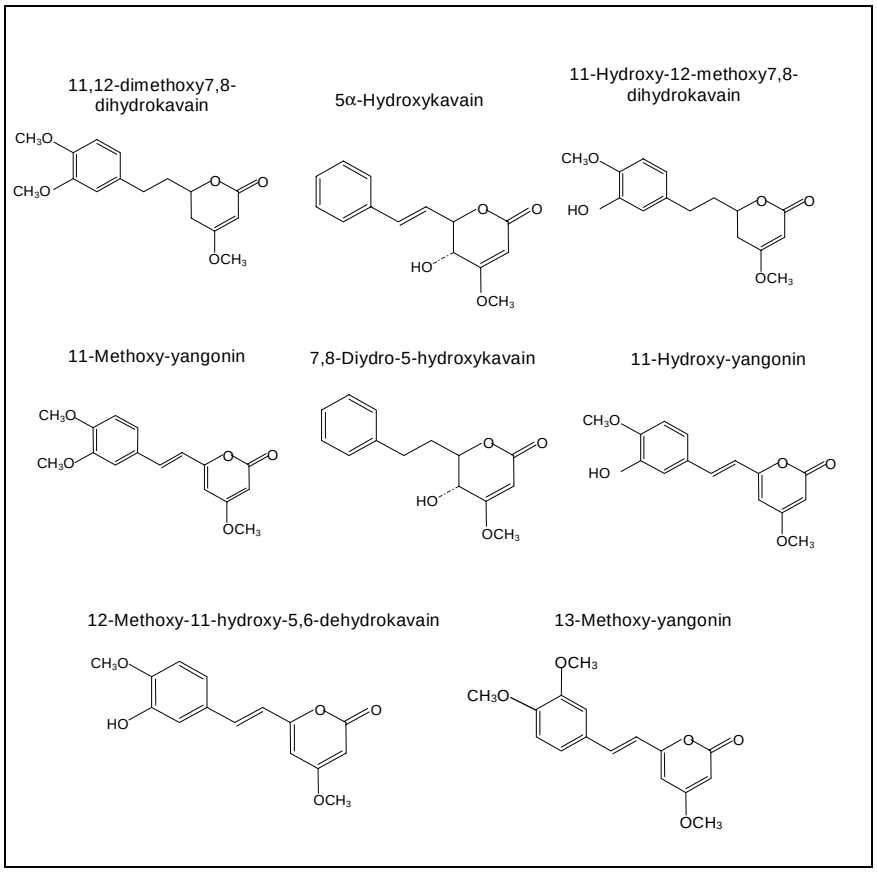


Figure 2: Chemical structures and names of the minor components of kava

Both major and minor kava lactones are present in variable concentrations in different parts of the plant [Smith 1983, Duffield et al. 1989]. The kava lactones are composed of 10.44 % of the lateral roots and 5.28 % of the dried rootstock (average of six samples). Concentrations of kava lactones are typically highest in the lateral roots, and decrease progressively towards the aerial parts of the plant. Kavain is notably absent in the leaves of the kava plant [Duve 1981].

1.5.2 Kava alkaloids

Although it was known earlier that alkaloids were present in the roots [Lavialle 1889, Winzheimer 1908, Scheuer and Horingan 1959], all attempts at their isolation were unsuccessful until 1971 when two amides were reported in trace amounts [Achenbach and Karl 1971]. More recently, the alkaloid pipermethystine (Figure 3) was isolated and identified in kava leaves [Smith 1979]. This compound is also

present in small amounts in the stems and roots of the plant, but because of its instability, it previously had not been reported [Singh 1992, Kaul and Joshi 2001].

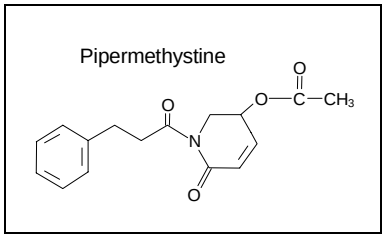


Figure 3: Chemical structure of the alkaloid pipermethystine

1.5.3 Chalcones and flavanones

Two pigment materials, flavokavain A and flavokavain B, were isolated from the kava rhizome by chromatography in a 0.04 % and 0.004 % yield, respectively [Hänsel et al. 1961, Harris and Combs 1968]. These chalcones are relatively devoid of any major biological activity and are believed to be responsible for the skin discoloration of people with chronic exposure to kava extracts [Sauer and Hänsel 1967]

1.6 Synthetic kavain

In contrast to the natural (+) kavain, the synthetic kavain (+/-) is a recemate and thus can be drastically different in the pharmacological properties of the enantiomares. Kavain, on hydrogenation, gave 7,8-dihydrokavain and on alkaline hydrolysis, kavaic acid. The first chemical synthesis of kavain (Figure 4) was already described by Fowler and Henbest [1950] and by Klostermans [1950].

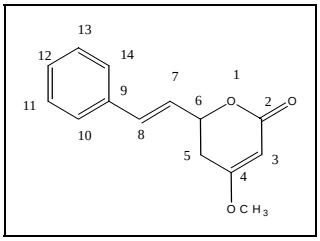


Figure 4: Chemical structure of kavain

However only the total synthesis by Israili and Smissman [1976] brought sufficient recovery for an investigation of the pharmacological effects of the several kava

constituents [Israili and Smissman 1976]. Kavain, 5,6-dehydrokavain and 7,8-dihydrokavain have been isolated by supercritical fluid extraction of kavalactones [Lopezavila et al. 1971].

1.7 Pharmacological properties of kava, kavain and other kava lactones

Early pharmacology of kava was based on clinical observations. In 1886 Lewin published his first monography on kava, which integrates data from the fields of different sciences, mainly ethnology, chemistry, pharmacology and toxicology.

Kava has enjoyed a long history of both social and clinical use in Polynesian culture and among the Aboriginal people of Arnhem Land in the Northern Territory of Australia. Kava has been used in these cultures for a variety of disorders. The active principles of the herb have revealed anticonvulsive, analgesic, anxiolytic and centrally acting muscle-relaxant activities [Lehmann 1998, Blumenthal et al. 1998, Czygan and Hiller 2002, Kaul and Joshi 2001].

Meyer conducted comprehensive studies on the physiological activity of the various kava lactones already during the 1950s and 1960s. This research and further studies determined that the main properties of kava lactones are: potentiation of barbituric narcosis [Klohs et al. 1959, Meyer 1962], analgesic effect [Brüggemann and Meyer 1963], local anaesthesia [Meyer 1964, Kretzschmar and Meyer 1965], muscular relaxation [Meyer 1965] and antimycotic activity [Hänsel et al. 1968].

Later on, the pharmacology of the kava lactones have been well studied by Hänsel 1968, Shuling 1973, Meyer 1979, Singh 1983, Cheng 1986, Duffield et al. 1986a, Duffield et al. 1986b, Lebot 1986.

The mild narcotic effect of kava was published 1924 by Schübel, and it has been asserted that kava paralyses the sensorial nerves, and later the straight muscles as well. Subsequent animal experiments [Brüggemann and Meyer 1963, Keledjian et al. 1988, Jamieson et al. 1989, Duffield et al. 1989 and Duffield et al. 1991] confirmed the antinociceptive effect of kava pyrones.

Hänsel and Beiersdorff proved the hypnotic effect of kava extract in animal experiments with rodents in 1959. To inhibit the EEG of sleep-wakefulness rhythm noticeably higher doses are necessary than for the muscle relaxation [Kretzschmar 1971 and Kretzschmar 1974].

Friedemann in 1996 had investigated the effect of a dry extract of the kava roots on patients who are suffering from anxiety, tension and agitation.

Today the most important pharmacological characteristics of kavain are anxiolytic effects which lead to a broad medical application. Kavain has to compete with neuroleptics, benzodiazepines and antidepressants in this area. In a study on anxiety patients [Lindenberg and Pitule-Schödel 1990] kavain has proven to be remarkably similar to oxazepam in its anxiolytic effect.

Kavain specifically inhibits the voltage-dependent Na^+ and Ca^+ channels in synaptosomes of the rat cerebral cortex [Gleitz et al. 1995, Seitz et al. 1997, Seitz et al. 1997, Schirrmacher et al. 1999 and Martin et al. 2000]. Kavain and dihydromethysticin posses anticonvulsant, analgesic, anxiolytic activities and antithrombotic activity [Lehmann 1989, Kinzler et al. 1991, Woelk et al. 1993, Walden et al. 1997 and Gleitz et al. 1997].

Backhaus et al. 1992 had studied the effects of kava extract and its pure isolates, methysticin and dihydromethysticin on ischemic brain damage in mice and rats. The kava extract and both its isolates protected against the cerebral damage to an extent comparable to the effect of the reference compound memantine. Kavain, dihydrokavain and yangonin failed to show this protection. The neuroprotective activity of kava may be due to methysticin and dihydromethysticin.

1.8 Mechanisms of action

Several studies have focused on the possible mechanisms of actions of kava and its constituents on the central nervous system. Davies 1992 were among the early investigations who studied the interaction of kava extract and its pure isolates with the GABA and benzodiazepine binding sites in the murine cerebral synaptosomal membrane receptors. The allosteric influence of kava extract on the GABA-A receptor-complex is similar to benzodiazepines [Jussofie et al. 1994, Friedemann 1996 and Kaul and Joshi 2001].

Others have suggested the involvement of the ion channel in the mediation of kavain effects on the central nervous system. Inhibition of the voltage gated Na^+ channel to induce muscle relaxation were also reported [Kretzschmar 1995, Gleitz et al. 1995 and 1996, Hänsel et al. 1999, Kohlenberg 1999]. Involvement of the serotonin 1-A

receptor in the medication of the central activity of kavalactones has been proposed by Walden et al. 1997. Since the serotonin receptors are implicated with anxiety, the anxiolytic activity of kava may involve the blockade of these receptors [Walden et al. 1997]. Recently Schmidt and Ferger et al 2001 reported about the beneficial action of kavain as a highly significant neuroprotective agent against MPTP induced Parkinson's disease.

According to all these mechanisms of action of kava and its products, it is difficult to specifically pinpoint how kava acts. Until and unless all of the individual bioactive constituents have been pharmacologically characterised, a large volume of controversial and widely spread data is likely to continue to accumulate.

1.9 Clinical studies

In a double-blind crossover study, volunteers were given by turns oxazepam and kava extract and a comparison of the two treatments was made in their respective effects on the volunteers recognition and memory [Munte et al. 1993,Lehmann et al. 1989, Kinzler et al. 1991 and Wurthmann et al. 1996]. The Hamilton Anxiety Scale (HAMA) for assessing main outcome variables, the Clinical Global Impression (CGI) for assessing secondary target variables and the Adjective Check List (ACL) were used to assess therapeutic efficacy of the treatment with kava WS 1490 (Laitan®). Both HAMA and CGI data showed a significant reduction of anxiety syndromes [Lehmann et al. 1989, Volz and Kieser 1997, Lehmann 1998, Scherer 1998, Pittler and Ernst 2000].

1.10 Toxicological properties of kava, kavain and other kava lactones

1.10.1 Kava dermopathy

The effect of chronic kava drinking on the skin has been mentioned and discussed in many reports. Beechey 1831, Thomsin 1908, Fornander 1916-1920 and Titcomb 1948 have done many studies on correlation between the heavy use of kava and the adverse effects on the skin [Kaul and Joshi 2001, Czygan and Hiller 2001, Hölzl et al. 1994, Hänsel 1996 and Singh 1992]. The skin of the kava user becomes dry and is

covered with scales, especially the palms of the hand, the soles of the feet and the forearms, back and shins. This skin reaction is known as kava dermopathy. It is an acquired reversible ichthyosis or scaly skin eruption, developing and persisting over several months or years. On the Fiji islands this lesions are called „kani“, and are thought to be combined with a prolonged and excessive kava consumption. It generally appears as a shiny and scaly skin [Norton and Ruze 1998]. Kava dermopathy is also believed to be related to interference with the cholesterol metabolism [Norton and Ruze 1998 and Jappe et al. 1998]. A dermatomyositis like illness following kava ingestion by white woman was examined [Guro-Razuman et al. 1999]. Frater postulated 1976 that the typical symptoms of vitamin-B mal absorption could be treated with a specific diet, and the reduction of the kava consume. Ruze [1990] proved in his study on kava consumers in Tonga, that the skin damage due to excessive kava consume can be treated by administration of niacin.

1.10.2 Other toxicological effects of heavy kava usage

Heavy consumption of kava has been reported to lead to adverse health effects in some Australian Aborigines. Mathews and co-workers [1988] published a pilot study on the health of 39 kava users and 34 non-users in an aboriginal community. Of all kava users, twenty respondents were very heavy kava users (mean consumption 440 g/week), 15 were heavy users (310 g/week) and 4 were occasional users (100 g/week). Kava users were more likely to complain about poor health and a puffy face, and were more likely to have a typical scaly rash and slightly increased patellar reflexes. The very heavy users of kava were generally 20 % underweight, and their γ-glutamyltransferase were greatly increased. Albumin, plasma protein, urea and bilirubin levels were decreased in kava users, and high density lipoprotein cholesterol levels were increased. Kava users were more likely to show haematuria, and had urine which poorly acidified with low specific gravity. The use of kava was also associated with an increased red cell volume, with a decreased platelet volume and lymphocyte count [Mathews et al. 1988]. Schelosky et al. [1995] reported the development of clinical signs suggestive of central dopaminergic antagonism in four patients who took kava preparations for anxiety.

1.10.3 Toxicological studies of D,L-kavain on animals

Acute and chronic toxicity studies with D,L-kavain were carried out on mice. These studies revealed that an LD_{50} after an oral uptake is not available. The LD_{50} value for mice is higher than 6000 mg/kg [Klinge Pharma 1995].

The effect of kavain was investigated in animal by application of oral doses to rats and dogs over 13 weeks (the rats doses: 50, 200 and 800 mg/kg/day; and for dogs 5, 50, 250 and 500 mg/kg/day) as well as over 26 weeks (the rats doses: 80, 240 and 720 mg/kg/day; and for dogs: 50, 150 and 450 mg/kg/day). With rats, in both chronic toxicity experiments, the respectively highest dosages lead to decreased weight gains, increased water consumption as well as liver and kidney weight gains. This could be interpreted as an early sign for kavain liver toxicity, which is discussed in the following. There were two deaths in the highest dosage group of the 26-weeks study, but due to missing toxicity symptoms and histological alteration, the death of the two rats could not be a result of kavain toxicity. In the long term experiment the liver weight gains and histological imposed reports (small to moderate nuclear polymorphs of the Parenchyma cells) were interpreted as a functional stress of the liver without pathological substrate. With dogs, dosages starting from 150 mg/kg in conjunction with a reduced food consumption lead to delayed weight gains. The animal urine analysis results showed no biochemical or haematological changes [Klinge Pharma 1995].

1.11 Kava lactones metabolism after consumption of kava extracts

Identification of a complex mixture of metabolites and unchanged kava lactones in human urine, following ingestion of kava prepared by the traditional method of aqueous extraction of commercial kava roots, was done by Duffield et al. 1989, Scheline 1991 and Johnson et al. 1991. The nine kava lactones identified were 7,8-dihydrokavain, kavain, 5,6-dehydrokavain, tetrahydroyangonin, 7,8-dihydromethysticin, 11-methoxytetrahydroyangonin, yangonin, methysticin and dehydromethysticin. 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 observed.

Rasmussen et al. 1979 investigated the metabolism of the enolidpyrones (= 5,6-dihydro-α-pyrones) 7,8-dihydrokavain, kavain and methysticin, and the dienolidpyrone 7,8-dihydroyangonin and yangonin in rats after oral application of some kava pyrones. The metabolic products in rats of each kava lactone were described as the following:

Kavain: although lower amounts of urinary metabolites were excreted following kavain administration, both hydroxylated and ring-opened products were formed. Metabolites identified included 12-hydroxybenzoic acid, 4-hydroxy-6-phenyl-5-hexene-2-one, hippuric acid, 4-hydroxy-6-hydroxyphenyl-5-hexene-2-one; 12-hydroxydihydrokavain, hydroxykavain, 12-hydroxykavain and 12-hydroxy-5,6-dehydrokavain (Figure 5).

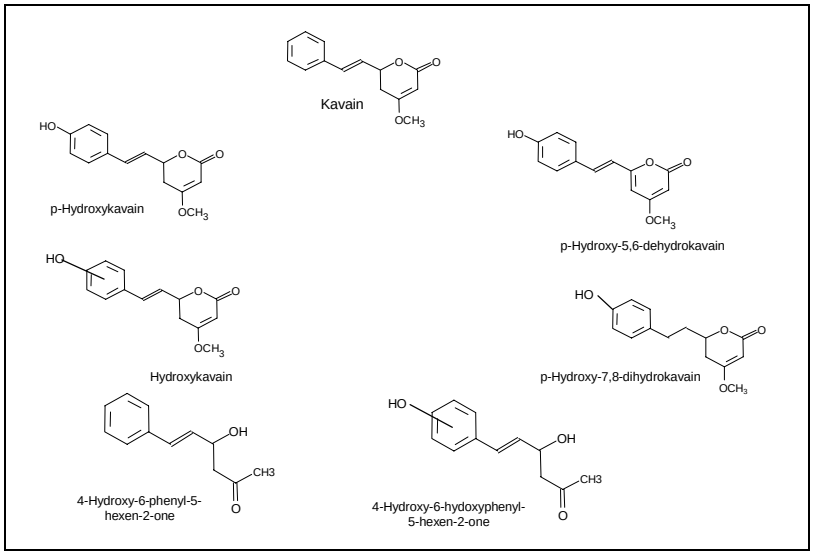


Figure 5: Major urinary metabolites of kavain in the rat [Rasmussen et al. 1979]

Two metabolites were not identified. The remaining derivatives are obviously monohydroxylated derivatives of kavain or its reduced or dehydrogenated derivatives. Large amounts of unchanged compound were identified in the faeces.

7,8-Dihydrokavain: approximately half of an oral dose of dihydrokavain (400 mg/kg) was recovered as metabolites in the urine in 48 h. A nearly 2:1 ratio between hydroxylated and ring opened products was observed.

7,8-Dihydrokavain metabolites are: 4-hydroxy-6-phenylhexen-2-one, 4-hydroxy-6-hydroxy-phenylhexen-2-one, 8-hydroxy-dihydrokavain, hydroxy-dihydrokavain, 12-hydroxy-dihydrokavain, and dihydroxy-dihydrokavain

(Figure 6). Small amounts of unchanged dihydrokavain were found in the faeces. No other metabolites were identified in faeces or in 0 to 22 h bile samples.

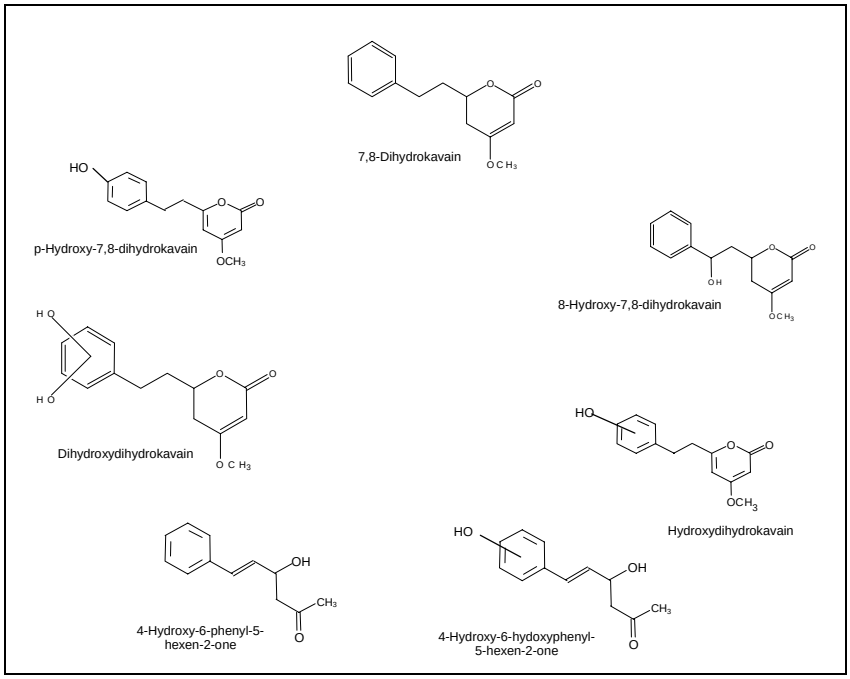


Figure 6: Major urinary metabolites of 7,8-dihydrokavain in rats [Rasmussen et al. 1979]

Methysticin: small amounts of two metabolites were detected in 0 to 48 h urine. Methysticin urinary metabolites are 11,12-dihydroxykavain and 11,12-dihydroxy-7,8-dihydrokavain (Figure 7). The absence of ring opened metabolites from methysticin was unexpected regarding their formation from both of the other 5,6-dihydro-α-pyrones studies. Unchanged methysticin was identified in faeces.

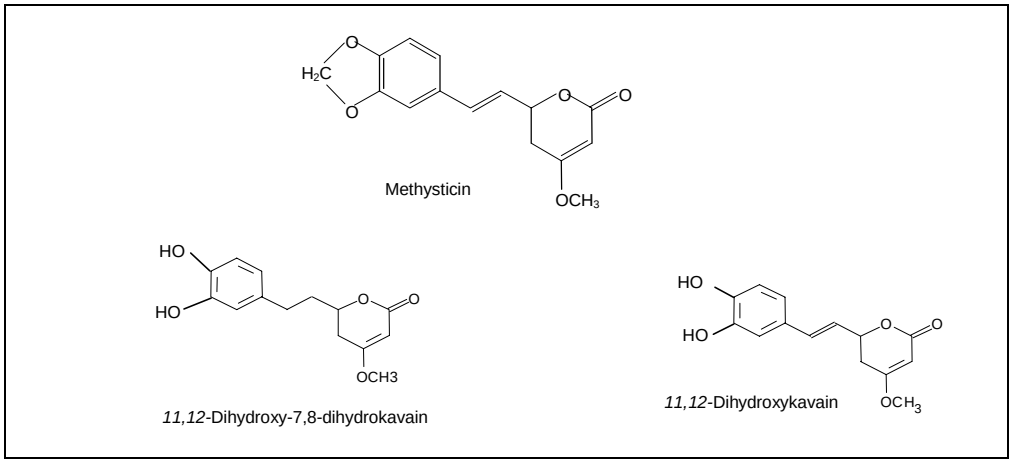


Figure 7: Major urinary metabolites of methysticin in rats [Rasmussen et al. 1979]

7,8-Dihydroyangonin: the major urinary metabolite of 7,8-dihydroyangonin was 12-hydroxy-5,6-dehydro-7,8-dihydrokavain, two minor metabolites were hydroxylated derivatives of this compound. They are defined as two dihydroxy-5,6-dehydro-7,8-dihydrokavain. However the positions of the second hydroxyl group (m, o or at C8) are uncertain (Figure 8). No ring opened products were detected.

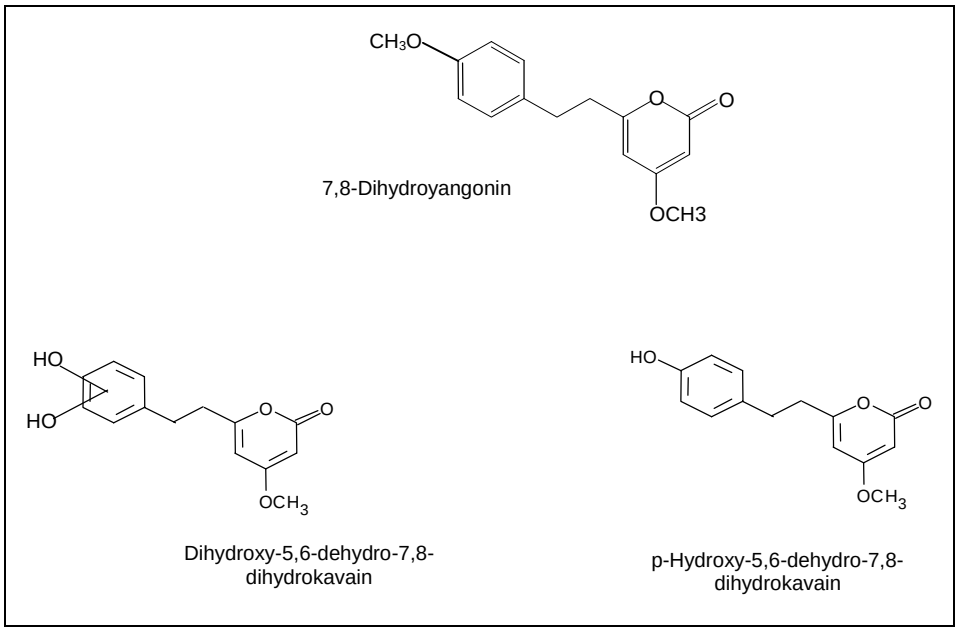


Figure 8: Major urinary metabolites of 7,8-dihydroyangonin in rats [Rasmussen et al. 1979]

Yangonin: relatively small amounts of yangonin metabolites were detected in urine. The three metabolites identified were formed via O-demethylation of the methoxy group; the metabolites were detected and identified as 12-hydroxy-5,6-dehydrokavain, 11,12-dihydroxy-5,6-dehydro-7,8-dihydrokavain and 11-hydroxy-5,6-dehydrokavain (Figure 9). The first and the third metabolites showed nearly the same mass spectrum, but the retention time of the first one is shorter. It is possible that both of them are geometrical isomers. No ring opened products were detected.

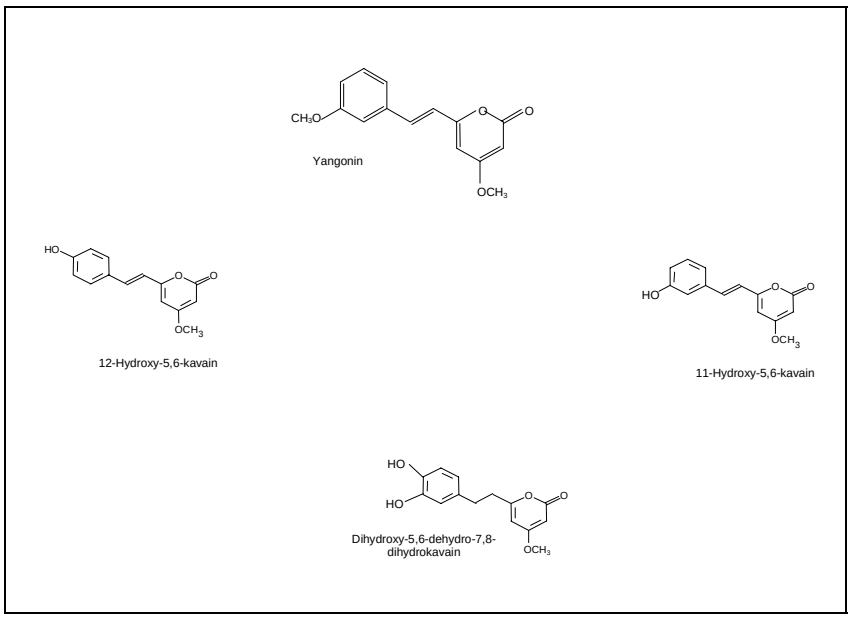


Figure 9: Major urinary metabolites of yangonin in rats [Rasmussen et al. 1979]

1.12 Kavain metabolism after consumption of synthetic D,L-kavain

Previous studies on the pharmacokinetics and pharmacodynamics of kavain [Klinge Pharma 1995] showed a maximum plasma concentration of about 18 ng/ml after an oral dose of 200 mg D,L-kavain. The initial resorption time of orally applied kavain in human subjects was about 15 min. The peak plasma concentration is reached in about 1.8 h. Kavain concentration in blood decreases with a half-life of about 9 h, the distribution phase lasts 3 to 5 h. The metabolite 12-hydroxykavain in form of its sulfate was detected in plasma samples. It reached its maximum 1.7 h after uptake, and showed a mean elimination half-life of 29 h. In urine, according to the given kavain dose, 5,6-dehydrokavain 0.3 %, free 12-hydroxykavain 1.0 % and conjugated 12-hydroxykavain (approx. 85 % sulfate and 15 % glucuronide) were found.

Another study was done on the D,L-kavain metabolism after oral administration [Köppel and Tenczer 1991]. In this study only the D,L-kavain urinary metabolites were discussed, like the free and the conjugated forms of 12-hydroxy-7,8-dihydrokavain, 12-hydroxykavain, 5,6-dehydrokavain and other decomposed products of kavain like decarboxylation products of kava acid (cinamaldehyde and cinnamylacetone).

1.13 Aim of the study

Up to this work, to our knowledge, no in vitro studies about the metabolism of kavain or kava lactones were published. Hepatic cells and microsomal incubation proved to be useful in metabolic profiling. One of the most important applications of in vitro systems is the identification of toxic metabolites [Sata and Kato 1982, Patel et al. 1991, Neuman et al. 1993, Hayes et al. 1995, Poon et al. 1995].

In comparison to isolated organs or organ slices, cell-cultures based on the liver carcinoma cell-line Hep G2 offer certain advantages as a system for studying human liver drug metabolism compared to other in vitro systems such as liver microsomes. Hep-G2 cells have proven to retain many of the specialised functions of normal liver parenchyma cells including expression of hepatocyte specific cell surface receptors [Schwartz 1981, Schwartz and Rup 1983, Huber et al. 1986]. They are easily available, and the variability in drug metabolising enzyme activities due to individual liver preparations is minimised with these cells which are intrinsically more reproducible.

The efficiency of cellular effects studied in Hep-G2 cells and its similarity to human liver was proven even when the toxic principle and metabolism have previously been unknown [Mahler et al. 1997]. Therefore Hep-G2 cell cultures were chosen as an in vitro model to study kavain metabolism in human liver. The specific objectives of this study were to compare the metabolic pathways of kavain in Hep-G2 cells and humans (blood, serum and urine) with the previously described rat urinary metabolites, and to identify and characterise major or yet unknown metabolites.

Furthermore highly sensitive analytical methods of identification and characterisation of main kavain metabolites in both systems (Hep-G2 cells and humans) should be developed, including effective extraction methods, which might be helpful in forensic inquiries like the examination of negative effects on driving under influence of kava.

2 Experimental

2.1 Preparation of Hep-G2-cells for the kavain metabolism study

2.1.1 Materials and chemicals

All compounds were of analytical grade. Dimethylsulfoxide, iodomethane, methanol, ethanol, hexane, acetone, ethyl acetate, acetonitrile, hydrochloric acid 25 %, ammonia solution 25 %, ammonium chloride, toluene, pyridine, isooctane, dichlormethane, disodium hydrogen phosphate, potassium dihydrogenphosphate, sodium bicarbonate, phosphoric acid, boric acid, sodium sulfate anhydrous, sodium hydroxide and water for chromatography: E. Merck, Darmstadt, Germany; N-methyl-N-trimethylsilyltrifluoroacetamide MSTFA: Macherey-Nagel, Düren, Germany; tetramethylammoniumhydroxide solution 25 % (TMAH), diethylether, acetic acid anhydride: Fluka, Neu-Ulm, Germany; D,L-kavain, 12-hydroxykavain, 12-hydroxy-7,8-dihydrokavain, 12-hydroxy-5,6-dehydrokavain and 6-phenyl-5-hexen-2,4-dion: were obtained from Klinge Pharma Ltd., München, Germany; d_5-diazepam: Promochem, Wesel, Germany.

CLEAN UP® Extraction columns CE C 18111 bonded silica, size 1 mg/ml from United Chemical Technologies. INC (Bristol PA, USA). The columns were used to perform the solid phase extraction procedure.

2.1.2 Buffers

Buffers and solutions were prepared as follows: buffer, pH 9: 1.78 g of disodium hydrogenphosphate (Na_2HPO_4) were dissolved in 100 ml water; borate buffer, pH 9: 835 ml borax solution (12.37 g boric acid solved in 100 ml 1 M NaOH with 0.05 M natriumtetraborate ad 1 l) were mixed with 165 ml 0.1 M HCl. The buffer solutions were adjusted with NaOH to pH 9.

2.1.3 Origin and Description of the Hep-G2-cells

Source

German Collection of Microorganisms and Cell Cultures, Department Human and Animal Cell Cultures (DSM, Braunschweig, Germany); ATCC-No.: ACC 180

Cell-Type

Human hepatocellular carcinoma

Origin

Established from the tumour tissue of a 15 year old Argentine boy with hepatocellular carcinoma in 1975

Protein-production

Alpha-fetoprotein, albumin, alpha2-macroglobulin, alpha1-antitrypsin, transferrin, alpha1-antichymotrypsin, hepatoglobin, ceruloplasmin, plasminogen, complement (C3, C4), C3 activator, fibrinogen, alpha1-acid glycoprotein, alpha2HS glycoprotein, ß-lipoprotein, retinal binding protein

Hepatitis

Hepatitis B virus genome negative

Morphology

Adherent, epithelial-like cells growing as mono-layers

Storage

Frozen at - 70 °C in 60 % RPMI 1640 Medium / 30 % fetal calf serum / 10 % dimethylsulfoxid (DMSO) at about 2-5x10^6 cells / 1.5 ml ampoule

Culture medium

90 ml RPMI 1640 Medium, 15 ml fetal calf serum and 2 ml of a solution with penicillin (10000 IU/ml) and streptomycin (10000 IU/ml) in RPMI 1640 medium

Culture condition

At 37 °C with 5 % CO_2

Subculture

Split confluent culture 1:2-1:4 every 3-6 days

Doubling time

ca. 40-60 h

Trypsin-EDTA-solution for cell harvest (Amimed)

0.05 % Trypsin 1:250, 0.02 % EDTA 10 mg/l phenol red in phosphate buffered saline

2.1.4 Production of the culture medium

All components are mixed shortly before their usage as culture media. Fetal calf serum and the penicillin / streptomycin – mixture are stored at - 20 °C and are thawed in a double boiler. RPMI 1640 medium, and the final culture supernatant are stored at 4 °C.

2.1.5 Aseptic technique for the preparation of Hep-G2 cell cultures

In general all liquid handling series and the preparations of the cell cultures are conducted solely under a sterile class 2 security workbench. Pipettes, vessels and materials are either obtained as sterile article in a sterile packaging or are sterilised with a vapour sterilizer at 120 °C for 30 min. The hands of the respective operator are washed with a solution of 70 % ethanol, hexetidine and hydrogen peroxide in water, then dried and covered with sterile gloves before any contact with the equipment for cell cultures and media.

2.1.6 Cell harvest for breeding with kavain endowed culture medium

The supernatant culture medium above the cells is carefully removed from the culture vessel (capacity 200 ml) with a pipette and replaced with 5 ml trypsin-EDTA-solution. Afterwards the culture is incubated for 5 min at 37 °C, which results in a destruction of the mono-layer and the removal of the cells from the surface of the culture vessel. After a short microscopic control the culture vessel is pushed several times against a soft material to separate the cells. Immediately 5 ml of the culture media is added to stop the effect of the enzyme. The medium is removed from the culture vessel, and filled into a centrifuge-tube where it is centrifuged for 5 min with 300 g at 24 °C. The

upper layer is decanted and discarded. The sediment is shaken and resuspended again with 1 ml of the culture media.

The number of the living cells in this suspension is determined using a Neubauer cell counter. Therefore, 10 µl cell suspension is mixed with 90 µl of a solution with trypan-blue. Merely the living (coloured) cells are counted with a 200 fold magnification using a phase contrast microscope. 250 µl of the cell culture suspension are incubated in a culture vessel with 20 ml culture media. For the cultivation in kavain endowed culture media, only cell cultures with at least 80 % mono-layer covering are used.

For endowment of the cell cultures with kavain, the kavain solution (kavain is dissolved in ethanol respectively ethanol / DMSO 1:1, and stored at room temperature) is mixed with 4 parts of a RPMI solution, and added drop wise into a strongly shaken centrifuge vessel with prewarmed cell free culture media through a solvent resistant sterile filter system. The culture media from a culture vessel with a closed mono-layer (surface covered to at least 80 %) cell culture is decanted and immediately replaced with the prewarmed kavain endowed culture media. Immediately afterwards the cell culture is incubated at 37 °C / 5 % CO_2.

The incubation is stopped (after an adequate period of time) by adding 3 parts of methanol to the cell culture medium. The culture vessel is shaken to suspend all cells. The suspension is afterwards centrifuged at 2000 g for 5 min. The methanol upper layer is removed and kept at - 20 °C until analysis (the samples could be stored up to 3 years).

2.2 Incubation of kavain with Hep-G2 cell cultures

The metabolism of kavain was determined with Hep-G2 cells at a single dose of 2 and 60 mg kavain in 200 ml culture medium. Cells were plated at 1×10^6 cells / 25 cm^2 / 5 ml culture medium. The cells were incubated at 37 °C for periods of 3 h, 10 h, 30 h and 70 h. A preparation of cell medium without cells was used for blank studies.

2.3 Extraction procedures

2.3.1 Fluid-fluid extraction

10 ml of supernatant culture medium (see Section 2.2) were transferred into a 100 ml separate funnel, mixed with 20 ml Na_2HPO_4 buffer (pH 9), and extracted with a mixture of 30 ml dichlormethane:ether (7:3, v/v). The lower organic phase was collected and evaporated in vacuum. The residue was dissolved in 2 ml methanol, and transferred into a glass vial. The methanol phase was dried under a stream of nitrogen, and redissolved in 50 µl methanol. 1 µl of the extract was analysed by GC/MS.

2.3.2 Solid-phase extraction (SPE) using Bond Elute C18 column

An alternative procedure for the extraction of kavain and metabolites was developed using solid phase re-extraction with bonded silica (Elute C18-columns): the organic fractions of fluid-fluid extraction (see Section 2.3.1) were combined, and their volume was reduced to dryness. The residue was redissolved in borate buffer (1 ml at pH 9) then applied to the SPE C18 column. The cartridge had been pre-conditioned by subsequent washing with 2 ml methanol, 2 ml water, 1 ml borate buffer, before 1 ml sample was added. The column was washed with 25 % methanol / water and dried by centrifugation (3000 rpm). Elution used methanol 2×0.75 ml. The eluate was evaporated to dryness using a cold stream of nitrogen. The residue was redissolved in methanol, and assayed for kavain and metabolites with and without derivatisation by GC/MS.

2.4 Derivatisation of kavain metabolites

2.4.1 Methylation

The dried extracts (fluid-fluid extraction and solid phase extraction Bond Elute C18) were mixed for 1 min (vortex-mixed) with 200 µl of a mixture of 50 µl TMAH (25 % in water) in 1 ml DMSO, 50 µl iodomethane were added and vortex-mixed for 1 min,

followed by 15 min of incubation in the dark at room temperature. 200 µl of 0.1 M HCl were added and vortex-mixed for 2 min followed by 2 min of dark incubation at room temperature. 1 ml isooctane was added and mixed for 3 min. Centrifugation at 14000 rpm was done for phase separation. 900 µl from the isooctane phase were collected and evaporated to dryness under a cold stream of nitrogen [Daldrup and Mußhoff 1995]. The residue was redissolved in 50 µl isooctane. 1 µl was analysed by GC/MS.

2.4.2 Silylation

The dried extracts (fluid-fluid extraction and solid phase extraction Bond Elute C18) were mixed with 220 µl mixture of 10 µl pyridine, 10 µl MSTFA and 200 µl isooctane (1 min, vortex-mixed) and incubated for 30 min at 90 °C [Daldrup and Mußhoff 1995]. 1 µl was analysed by GC/MS.

The reference substances III, V, IX and XIV were derivatised according to the above procedure.

2.5 GC/EI MS-method for the detection and identification of kavain and its metabolites

The analyses were performed with a Hewlett Packard GC/MS system: GC 5890; MSD 5970 equipped with a Hewlett Packard automatic liquid sampler HP 7673, chromatographic conditions were: column: HP-5 MS, 30 m length, ID 0.25 mm (0.25 µm film thickness); carrier gas: helium (pressure 70 kPa); split / purge off time: 2 min; injector temperature: 270 °C; transfer line temperature: 280 °C; temperature program: initial temperature 60 °C for 2 min, 4 °C / min to 200 °C for 2.5 min, 10 °C / min to 300 °C for 5 min maintained for 5 min (total run time: 50 min). The internal standard was d5-diazepam with RT 42.45 min. The mass spectrometer was used in electron impact (EI) mode at 70 eV. Scan Mode, m/z: 50-450.

2.6 Physical and spectrographic data of the detected metabolites

2.6.1 GC/EI MS and HPLC-DAD data base of kavain and its metabolites

The reference substances available for this study were: D,L-kavain (k), 12-hydroxykavain (III), 12-hydroxy-7,8-dihydrokavain (V), 12-hydroxy-5,6-dehydrokavain (IX) and 6-phenyl-5-hexene-2,4-dione (XV).

d,l-Kavain (K)

Molecular formula: $C_{14}H_{14}O_3$; MW: 230

HPLC *RT: 6.5 min*

UV: λ_{max} (nm) detected with the wavelength: 202.8; 247.4 nm, reference substance: 204.0; 246.2 nm and from the literature: 202 ; 245 nm [He et al. 1997], 245 nm [Hänsel et al. 1967]

GC/EI-MS: *RT*: 40.5 min; detected with mass fragments m/z (rel. int.): 230 $[M]^+$ (33), 202 $[M\text{-}CO]^+$ (48), 131 $[C_{10}H_{11}]^+$ (20), 115 $[C_9H_7]^+$ (23), 104 $[C_8H_8]^+$ (27), 98 $[C_5H_6O_2]^+$ (100), 91 $[C_7H_7]^+$ (38), 68 $[C_4H_4O]^+$ (75)

Kavain as reference has the mass fragments m/z (rel. int.): 230 $[M]^+$ (30), 202 (45), 131 (25), 115 (23), 104 (29), 98 (100), 91 (40), 68 (95)

Literature mass spectra data for kavain: m/z (rel. int.): 230 $[M]^+$ (27), 202 (32), 131 (9), 115 (7), 104 (22), 98 (95), 91 (40), 68 (100), 77 (18) [Köppel and Tenczer 1991]

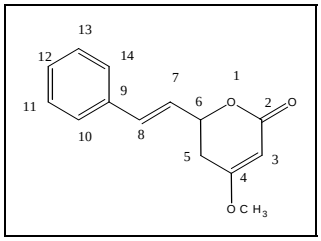


Figure 10: Chemical structure of D,L-kavain

12-Hydroxykavain (III)

Molecular formula: $C_{14}H_{14}O_4$; MW: 246

HPLC *RT: 4.5 min*

UV: λ_{max} (nm) detected with the wavelength: 205.2; 262,7 nm and reference substance: 207.0; 261.5 nm.

Trimethyl silyl ether of (III-TMS)

Molecular formula: $C_{17}H_{22}O_4Si$; MW: 318

GC/EI-MS: *RT*: 44.1 min; detected with mass fragments; m/z (rel. int.): 318 $[M]^+$ (40), 179 $[C_{10}H_{15}OSi]^+$ (100), 192 $[C_{11}H_{16}OSi]^+$ (95), 219 $[C_{13}H_{19}OSi]^+$ (50), 205 (15), 98 $[C_5H_6O_2]^+$ (48).

The mass spectra of the reference substance: *RT*: 44.1 min; m/z (rel. int.): 318 $[M]^+$ (50), 179 (100), 192 (98), 219 (45), 205 (25), 98 (46)

The mass spectra data from the literature were: m/z (rel. Int.): 318 $[M]^+$ (22), 274 (17), 273 (12), 220 (13), 219 (25), 192 (12), 179 (100), 177 (36), 128 (17), 127 (13) [Rasmussen et al. 1979]

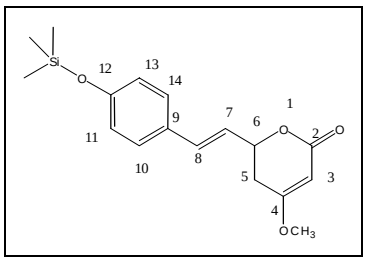


Figure 11: Chemical structure of trimethyl silyl ether of 12-hydroxykavain

12-Hydroxy-5,6-dehydrokavain (IX)

Molecular formula: $C_{14}H_{12}O_4$; MW: 244

HPLC *RT: 5.5* min

UV: λ_{max} (nm) detected with the wavelength: 219.5; 362.5 nm and the reference substance 216.9; 359.5 nm

Trimethyl silyl ether of (IX-TMS)

Molecular formula: $C_{17}H_{20}O_4Si$; MW: 316

GC/EI-MS: *RT*: 44.5 min; detected with the mass fragments; m/z (rel. int.): 316 $[M]^+$ (100), 245 $[C_{14}H_{13}O_4]^+$ (62), 288 (54), 301 $[C_{16}H_{17}O_4Si]^+$ (10), 273 $[C_{16}H_{17}O_4]$ (20), 179 $[C_{10}H_{15}OSi]^+$ (9), 115 (23), 135 (13)

The GC/EI-MS of the reference substance: *RT*: 44.5; has the mass spectra: m/z (rel int.): 316 $[M]^+$ (100), 245 (60), 288 (50), 301 (12), 273 (10), 179 (7)

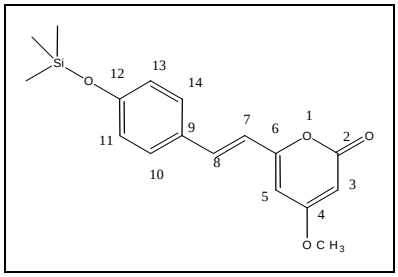


Figure 12: Chemical structure of trimethyl silyl ether of 12-hydroxy-5,6-dehydrokavain

12-Hydroxy-7,8-dihydrokavain (V)

Molecular formula: ($C_{14}H_{16}O_4$); MW: 248

HPLC *RT:* 4.3 min

UV: λ_{max}: detected with the wavelength: 193; 227 nm, reference substance: 194.6; 226.2 nm

Trimethyl silyl ether (V-TMS)

Molecular formula: $C_{17}H_{24}O_4Si$; MW: 320

GC/EI-MS: *RT*: 42.75 min, detected with the mass spectra m/z (rel. int.): 320 $[M]^+$ (30), 179 $[C_{10}H_{15}OSi]^+$ (100), 192 $[C_{11}H_{16}OSi]^+$ (20),.127 $[C_6H_7O_3]^+$ (35), 205 (65)

The GC/EI-MS of the reference substance: *RT*: 42.75; has the mass spectra: m/z (rel int.): 320 $[M]^+$ (35), 179 (100), 192 (40), 127 (35), 205 (65)

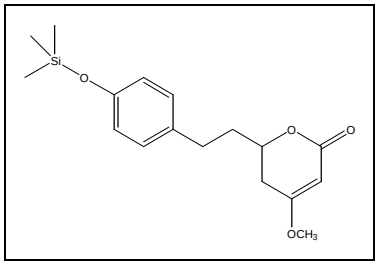


Figure 13: Chemical structure of trimethyl silyl ether of 12-hydroxy-7,8-dihydrokavain

6-Phenyl-5-hexen-2,4-dione (XV)

Molecular formula: ($C_{12}H_{12}O_2$); MW: 188

Trimethyl silyl ether of (XV-TMS)

Molecular formula: $C_{15}H_{20}O2Si$; MW: 260

GC/EI-MS: detected in its two isomers *RT*: 32.61 and 32.69 min, detected with the mass spectra: m/z (rel. int.) for isomer 1: 260 $[M]^+$ (34), 245 $[C_{14}H_{17}O_2Si]^+$ (98), 217 $[C_{13}H_{17}OSi]^+$ (100), 183 $[C_9H_{15}O_2Si]^+$ (76), 103 $[C_8H_7]^+$ (40), 115 $[C_9H_7]^+$ (30), 170 $[C_8H_{14}O_2Si]^+$ (32), 155 (60), 131 (65);

for isomer 2: 260 $[M]^+$ (33), 245 $[C_{14}H_{17}O_2Si]^+$ (100), 217 $[C_{13}H_{17}OSi]^+$ (98), 183 $[C_9H_{15}O_2Si]^+$ (60), 103 $[C_8H_7]^+$ (30), 115 $[C_9H_7]^+$ (25), 170 $[C_8H_{14}O_2Si]^+$ (32), 155 (54), 131 (52)

The reference substance has also two isomers with mass spectra; *RT*: 32.61 and 32.69 min; GC/EI-MS: m/z (rel. int.) for isomer 1: 260 $[M]^+$ (40), 245 (95), 217 (100), 183 (72), 103 (45), 115 (35), 170 (30), 155 (62), 131 (63); for isomer 2: 260 $[M]^+$ (35), 245 (100), 217 (93), 183 (65), 103 (36), 115 (30), 170 (32), 155 (55), 131 (59)

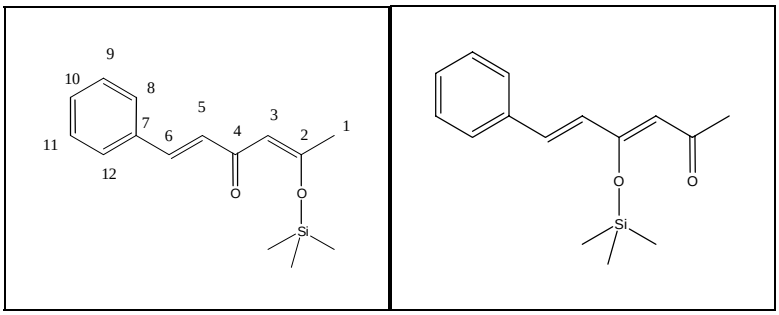


Figure 14: Chemical structure of trimethyl silyl ether of 6-phenyl-5-hexene-2,4-dione

5,6-dehydrokavain (VI)

Molecular formula: $C_{14}H_{12}O_3$; MW: 228

LC-MS RT: 14.32 min; UV: λ_{max} (nm): detected with the wavelength: 208, 255, 344 nm. The literature UV data were 208, 230, 255, 343 nm [He et al. 1997] and 231, 255, 344 nm [Hänsel et al. 1967]

GC/EI-MS: *RT*: 40.55 min, detected with mass spectra: m/z (rel. int.): 228 $[M]^+$ (100), 157 (45), 200 (35), 211 (20), 185 (19), 103 (10), 115 (9), 80 (20), 69 (31)

The literature GC/EI-MS data: m/z (rel. int.): 228 $[M]^+$ (100), 157 (36), 200 (24), 185 (11), 103 (37), 140 (43), 131 (12), 129 (16), 128 (16), 114 (18), 103 (23), 77 (40), 69 (48) [Köppel and Tenczer 1991]

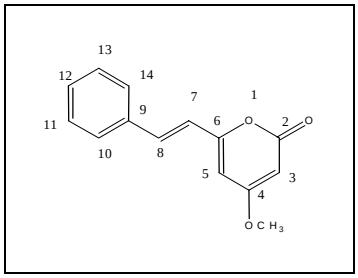


Figure 15: Chemical structure of *5,6-dehydrokavain*

2.6.2 EI-MS data of kavain metabolites from the literature

12-Hydroxybenzoic acid (XVI-TMS)

GC/EI-MS: m/z (rel. int.): 224 (100), 210 (30), 209 (92), 193 (56), 179 (12), 177 (52), 149 (35), 135 (66) [Rasmussen et al. 1979]

Hippuric acid methyl ester

GC/EI-MS: m/z (rel. int): 193 (8), 161 (5), 134 (14), 105 (100) [Rasmussen et al. 1979]

Trimethyl silyl ether of 4-hydroxy-6-phenyl-hex-5-ene-2-one (XIV- 2 x TMS)

GC/EI-MS: m/z (rel. int.): 350 $[M]^+$ (7), 335 (10), 294 (24), 293 (100), 219 (12), 203 (16), 193 (18), 179 (42), 143 (21), 115 (28) [Rasmussen et al. 1979]

Trimethyl silyl ether of hydroxy-kavain (I-TMS)

GC/EI-MS: m/z (rel. int.): 318 $[M]^+$ (12), 305 (5), 274 (32), 273 (31), 243 (15), 227 (12), 212 (25), 192 (26), 180 (16), 179 (100), 177 (17), 127 (8) [Rasmussen et al. 1979]

Trimethyl silyl ether of 12-hydroxykavain (III-TMS)

GC/EI-MS: m/z (rel. int.): 318 $[M]^+$ (22), 274 (17), 273 (12), 220 (13), 219 (25), 192 (12), 179 (100), 177 (36), 128 (17), 127 (13) [Rasmussen et al. 1979]

Trimethyl silyl ether of 12-hydroxydihydrokavain (IV-TMS)

GC/EI-MS: m/z (rel. Int.): 320 $[M]^+$ (24), 305 (2), 206 (5), 205 (30), 192 (7), 180 (21), 179 (100), 128 (10), 127 (18) [Rasmussen et al. 1979]

Trimethyl silyl ether of 12-hydroxy-5,6-dehydrokavain (IX-TMS)

GC/EI-MS: m/z (rel. int.): 316 $[M]^+$ (100), 301 (8), 288 (26), 273 (17), 245 (30), 179 (34), 149 (2), 137 (15), 135 (22), 125 (15) [Rasmussen et al. 1979]

Cinnamyl-acetone (XI)

GC/EI-MS: m/z (rel. int.): 189 $[M]^+$,104 (100), 121 (90), 77 (50), 133 (10), 91 (12), 107 (7) [Köppel and Tenczer 1991]

4-Hydroxy-cinnamyl-acetone (XI-TMS)

GC/EI-MS: m/z (rel. int.): 190 $[M]^+$ (22), 172 (11), 133 (28), 132 (30), 131 (29), 129 (8), 115 (22), 105 (23), 104 (100) [Köppel and Tenczer 1991]

4-Oxy-cinnamyl-acetone (XIII)

GC/EI-MS: m/z (rel. int.): 188 $[M]^+$ (100), 127 (90), 77 (50), 115 (40), 121 (37), 91 (20), 173 (15), 131 (2), 141 (3) [Köppel and Tenczer 1991]

6-phenyl-3-hexen-2-one (XIV)

GC/EI-MS: m/z (rel. int.): 174 $[M]^+$ (48), 159 (4), 141 (47), 91 (100), 117 (28), 104 (12), 92 (32) [Köppel and Tenczer 1991]

Unidentified metabolite (XVII)

GC/EI-MS: m/z (rel. int.): 380 $[M]^+$ (15), 365 (6), 324 (30), 323 (100), 290 (37), 247 (64), 233 (24), 217 (35), 179 (35), 143 (34), 115 (44) [Rasmussen et al. 1991]

The GC/MS analyses were carried out with and without derivatisation steps using also Selected Ions Monitoring (SIM) mode:

The SIM mode method was written according to the full scan of each analyte and according to those data from the previous literatures [Rasmussen et al. 1979, Duffield et al. 1989, Köppel and Tenczer 1991 and He et al. 1996]. The selection was based on the molecular ion, base peak and the prominent fragments of each metabolites.

For the none derivatised substances like kavain, 5,6-dehydrokavain, cinnamyl acetone, 4-oxy-cinnamyl acetone and others, the following masses were selected: m/z 69, 77, 86, 91, 98, 104, 107, 114, 115, 121, 127, 131, 133, 140, 141, 173, 174, 179, 185, 188, 189, 212, 228, 230.

For the metabolites which are derivatised by methylation like kava acid and some other endogenous substances like benzoic acid and hippuric acid : m/z 55, 68, 69, 77, 91, 114, 115, 121, 134, 147, 151, 152, 159, 161, 170, 185, 187, 230, 244, 260.

For the metabolites which are derivatised by silylation like 12-hydroxykavain, 12-hydroxy-5,6-dehydrokavain, 12-hydroxy-7,8-dihydrokavain, 6-phenyl-hex-5-ene-2,4-dione and some other unidentified metabolites from the previous literatures: m/z 105, 115, 127, 128, 134, 135, 143, 177, 179, 185, 192, 193, 205, 224, 230, 245, 247, 260, 273, 293, 302, 316, 318, 320, 323, 380.

2.7 Identification of kavain and its metabolites in human samples

2.7.1 Study design

The experimental study is based on many self medication trials.

2.7.1.1 Guide the strategy of the metabolism study

Kavain was administrated in a single oral dose of 200 mg (one Neuronika® capsules each of 200 mg D,L-kavain) corresponding to 1.9 mg/kg (body weight). Blood and urine samples were collected before and after the oral uptake of kavain. 10 ml of blood divided into 2 glass tubes, 5 ml each. One of the glass tubes was centrifuged at 3000 rpm to collect the serum samples. The blood samples have been taken at the time course of 45 min, 1,5, 3, 6, 12 and 24 h. The urine samples were collected 2, 5, 12, 24 h after the oral dose. This trial was done twice

2.7.1.2 The experiment trial using the GC/MS method

Kavain was administrated in a single oral dose of 800 mg (four Neuronika® capsules each of 200 mg D,L-kavain) corresponding to 6.9 mg/kg (body weight).

Blood and urine samples were collected before and after the oral uptake of kavain. 10 ml of blood divided into 2 glass tubes, 5 ml each. One of the glass tubes was centrifuged at 3000 rpm to collect the serum samples. The blood samples have been taken at the time course of 0.5 h, 8 h and 24 h. The urine samples were collected 2, 5, 9, 12, 24 h after the oral dose.

An aliquot of each urine (5 % of the urine) was collected separately, and analysed for the determination of kavain and its metabolites at each time point. The remnants of each urine (95 % of each urine) were collected as 24 h urine. Blood, serum and urine samples were stored at - 20 °C until the analyses (storage periods up to 3 years).

2.7.1.3 The experiment trial using the HPLC-DAD method

Kavain was administrated in a single oral dose of 800 mg (four Neuronika® capsules each of 200 mg D,L-kavain) corresponding to 7.7 mg/kg (body weight).

Blood and urine samples were collected before and after the oral uptake of kavain. 10 ml of blood divided into 2 glass tubes, 5 ml each. One of the glass tubes was centrifuged at 3000 rpm to collect the serum samples. The blood samples have been taken at the time course of 15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 240 minutes (15 min up to 4 h), urine samples were collected after 2, 4, 8, 18, 24 h after the 800 mg of kavain oral dose. An aliquot of each urine (5 % of the urine) was collected separately, and analysed for the determination of kavain and its metabolites at each

time point. The remnants of each urine (95 % of each urine) were collected as 24 h urine. Blood, serum and urine samples were stored at - 20 °C until the analyses (storage periods up to 3 years). This second trial of self medication used the HPLC-DAD method for the kinetic study of 12-hydroxykavain.

2.7.2 Materials and chemicals

The materials and chemicals used in this study were the same as mentioned in Section 2.1.1. In addition formic acid, ammonium formate (E. Merck, Darmstadt, Germany), brotizolam (Promochem, Wesel, Germany), and β-glucuronidase and aryl sulfatase from Helix pomatia (EC 3.2.1.31 type H-1, Sigma Deisenhofen, Germany), β-glucuronidase from Escherichia coli (EC 3.2.1.31 Type K 12, Roche Diagnostics Ltd., Mannheim, Germany) were used.

2.7.3 Preparation of standards

2.7.3.1 Stock solutions of the reference substances

d,l-Kavain (K), 12-hydroxykavain (III), 12-hydroxy-7,8-dihydrokavain (V), 12-hydroxy-5,6-dehydrokavain (IX) and 6-phenyl-5-hexen-2,4-dion (XV) were dissolved in methanol at concentrations of 0.1 % (1 µg/µl) and stored at 4 °C.

2.7.3.2 Internal standards for GC/MS, HPLC and LC/MS analyses

d_5-Diazepam in methanol 0.001 % (10 ng/µl) was used as internal standard for the GC/MS analysis.

Brotizolam in methanol 0.01 % (100 ng/µl) was used as internal standard for the HPLC and LC/MS analysis.

2.7.4 Buffers

Buffers and solutions were as follows:

Solution A: 1.19 g of disodium hydrogen phosphate (Na_2HPO_4) were dissolved in 100 ml water.

Solution B: 0.91 g of potassium dihydrogen phosphate (KH_2PO_4) were dissolved in 100 ml water.

Buffer, pH 5.5: saturated sodium dihydrogen phosphate (NaH_2PO_4) in water.

Buffer, pH 7: prepared by mixing 60 ml solution A with 40 ml solution B.

Buffer, pH 9: 1.78 g of disodium hydrogenphosphate (Na_2HPO_4) were dissolved in 100 ml water.

Elution system:

156 g acetonitrile were mixed with 344 g buffer (4.8 g H_3PO_4 85 % and 6.66 g KH_2PO_4) were completed with water to 1 L, pH 2.3.

2.7.5 Determination of kavain and its metabolites in blood and urine using the GC/MS method

2.7.5.1 Selected Ion Monitoring mode (SIM)

A SIM mode method was used for the detection of free kavain and four of its metabolites after silylation in human samples. The following fragments were selected:

Kavain (K): (m/z) 230 $[M]^+$, 202, 185, 98 (base peak), 68.

Trimethyl silyl of 12-hydroxykavain (III) m/z, 318 $[M]^+$, 179 (base peak), 192 and 219.

Trimethyl silyl of 12-hydroxy-7,8-dihydrokavain (V) m/z 320 $[M]^+$, 179 (base peak), 205, 127 and 164.

Trimethyl silyl of 12-hydroxy-5,6-dehydrokavin (IX) m/z 316 $[M]^+$(base peak), 288, 245 and 273

Trimethyl silyl of 6-phenyl-5-hexen-2,4-dion (XV) isomer 1: m/z 260 $[M]^+$, 217 (base peak), and 245; ismer 2: 260 $[M]^+$, 217 and 245 (base peak)

Instrumentation and method used see page 23, chapter 2.5.

2.7.5.2 Method validation

Blank serum samples were spiked with kavain, 12-hydroxykavain, 12-hydroxy-7,8-dihydrokavain and 12-hydroxy-5,6-dehydrokavain at concentrations of 20, 200, 1000,

2000 and 5000 ng/ml for the linearity test and at a concentration of 500 ng for the precision (within day and between days).

1 ml spiked serum samples were mixed with 10 µl 0.01 % d5-diazepam (as internal standard) and 2 ml buffer (pH 9). The samples were extracted with 3 ml of dichlormethane:diethylether (7:3, v/v), vortex-mixed for 5 min and centrifuged at 3000 rpm for 10 min. The aqueous layer was discarded. The organic phase was transferred into a high recovery glass vial, and evaporated to dryness (N_2 stream). The extract was analysed with and without derivatisation (see page 22, chapter 2.4).

2.7.5.3 Proof of glucuronided kavain metabolites in blood, serum and urine

Samples (1 ml) of blood, serum and urine were mixed with 100 µl buffer (pH 7) and 200 units of ß-glucuronidase from E. coli. The samples were incubated for 5 min at room temperature. Blood and urine were extracted according to the procedure mentioned on page 22 chapter 2.3.

2.7.6 Determination of kavain and its metabolites in human blood, serum and urine using the HPLC-DAD method

2.7.6.1 Method validation

Blank serum samples were spiked with kavain, 12-hydroxykavain, 12-hydroxy-7,8-dihydrokavain and 12-hydroxy-5,6-dehydrokavain. Due to the blood concentrations, the calibrators for kavain were at concentrations of 5, 10, 20, 40, 60 and 100 ng/ml and for the metabolites at concentrations of 50, 100, 200, 500 and 600 ng/ml. For the calculation of the precision of the method, serum samples with final concentrations of 5 and 40 ng/ml for kavain and 50 and 500 ng/ml for its metabolites were prepared.

The spiked serum samples were mixed with 10 µl 0.01 % brotizolam as internal standard, 100 µl buffer (pH 9) and 1 ml of dichlormethane: diethylether (7:3, v/v), vortexed for 10 min and centrifuged at 14000 rpm for 10 min. The aqueous layer was discarded. The organic phase was transferred into a high recovery glass vial and evaporated to dryness (N_2 stream). The extract residues were reconstituted in 50 µl methanol, 10 µl were analysed by HPLC-DAD.

2.7.6.2 Proof of glucuronided and sulfateted kavain metabolites in blood, serum and urine

Samples (1 ml) of blood, serum and urine were mixed with 100 µl buffer (pH 7) and 200 units of ß-glucuronidase from E. coli. The samples were incubated for 5 min at room temperature.

Samples (1 ml) of blood, serum and urine were mixed with 100 µl buffer (pH 5.5) and 200 units of ß-glucuronidase and arylsulfatase from H. pomatia. The samples were incubated in a water bath at 45 °C for 24 h.

After the enzymatic hydrolysis, samples were extracted as in Section 2.7.6.1. For the determination of kavain metabolites in their unconjugated form blood, serum and urine samples were extracted without enzymatic treatment.

2.7.6.3 Instrumentation used for High performance liquid chromatography

2.7.6.3.1 Isocratic HPLC- Diode-array Detector (DAD)

The analyses were performed with a Waters 2690 separations module with Waters 996 PDA Detector. Chromatographic condition were: Reversed-phase LiChrospher 60, RP-select B column 250 mm X 4.0 mm ID, particle size 5 µm (E. Merck, Darmstadt) with isocratic conditions (acetonitrile (31 %, w/w) / phosphate buffer pH 2.3) at a flow rate of 1 ml/min; column temperature was set to 27 °C. Chromatograms were recorded at 190 to 420 nm with a resolution of 1.2 nm [Daldrup et al. 1982]. The reference wavelength for kavain (246 nm), 12-hydroxykavain (262 nm), 12-hydroxy-7,8-dihydrokavain (226 nm) and 12-hydroxy-5,6-dehydrokavain (226 nm) were selected.

2.7.6.3.2 Liquid chromatography/mass spectrometry (LC/MS)

The following instrumentation was used: an API 365 triple-quadrupole mass spectrometer from Applied-Biosystems / Sciex (Langen, Germany), a Pentium PC with Analyst 1.1 software. Gradient elution using two pumps LC10AD (Shimadzu, Duisburg, Germany) and a reversed-phase column (Synergy polar-RP hexyl-propyl, polar endcapped), 2 mm i. D. x 150 mm, 3 µm particle size (Phenomenex, Aschaffenburg, Germany) with a synergy polar-RP guard cartridge (2 mm i. D. x 4 mm) (Phenomenex). This HPLC system was coupled without split to the API 365

using a Turbo IonSpray^{TM} source using heated nitrogen (3 l/min, 400 °C) as turbo-gas (for drying of the spray). Deionized water (< 0.1 µS from a cartridge-deionizer, Memtech, Moorenweis, Germany), gradient grade acetonitrile, 25 % aqueous ammonia and formic acid (analytical grade, Merck) were used for HPLC solvents or for dissolving drug standards.

For HPLC, the following gradient was used with solvent A (1 mM ammonium formate, 0.1 % formic acid, pH 3) and solvent B (acetonitrile / water, 95:5, v/v; containing 0.1 % formic acid and 1 mM ammonium formate) using a total flow rate of 0.25 ml/min: 0 to 1 min: 5 % B; 1 to 5 min: 5 % to 30 % B linear; 5 to 15 min: 30 % to 70 % B linear; 15 to 19 min: 70 % to 95 % B linear; 19 to 22 min: 95 %; B; 22 to 24 min: 95 % to 5 % B linear; 24 to 28 min: 5 % B (for equilibration). For the ESI/CID-spectra of reference compounds a RP-C18 column was used (XTerra C18, 100 x 2 mm, 3.5 µm particle size, Waters, Eschborn, Germany) with the same gradient but with solvent A (0.1 % formic acid, 1 mM ammonium formate) and solvent B (acetonitrile). Ionisation was performed using a needle voltage of 5250 V (positive mode). 10 µl aliquots of urine extract (dissolved in HPLC-solvents A:B, 80:20, v/v) were injected.

For the acquisition of reference spectra, 200 ng of the reference compounds (kavain and four metabolites see 2.7.3.1) had been injected in previous experiments using single-quadrupole mode (Q1-scan) with a scan-range of 50 to 550 amu and a looped experiment with orifice voltage switching (20, 50 and 80 V) between each scan, a dwell-time of 0.2 msecs and a step-size of 0.1 amu [Weinmann et al. 1999]. This caused a total scan-time for the looped experiment of 6 seconds. Mass resolution had been set to 0.7 $\pm$ 0.1 amu (peak width at half height) using polypropyleneglycol for mass axis calibration.

2.8 Retention times *(RT),* mass and UV spectra of kavain and four metabolites

2.8.1 GC EI/MS method

All analyses were run in the EI mode (70 eV). The identification of kavain derivatives was based on reference mass spectra. The GC/MS chromatogram and mass spectra of kavain and 4 of its metabolites are shown at concentration of 100 ng (Figure 16).

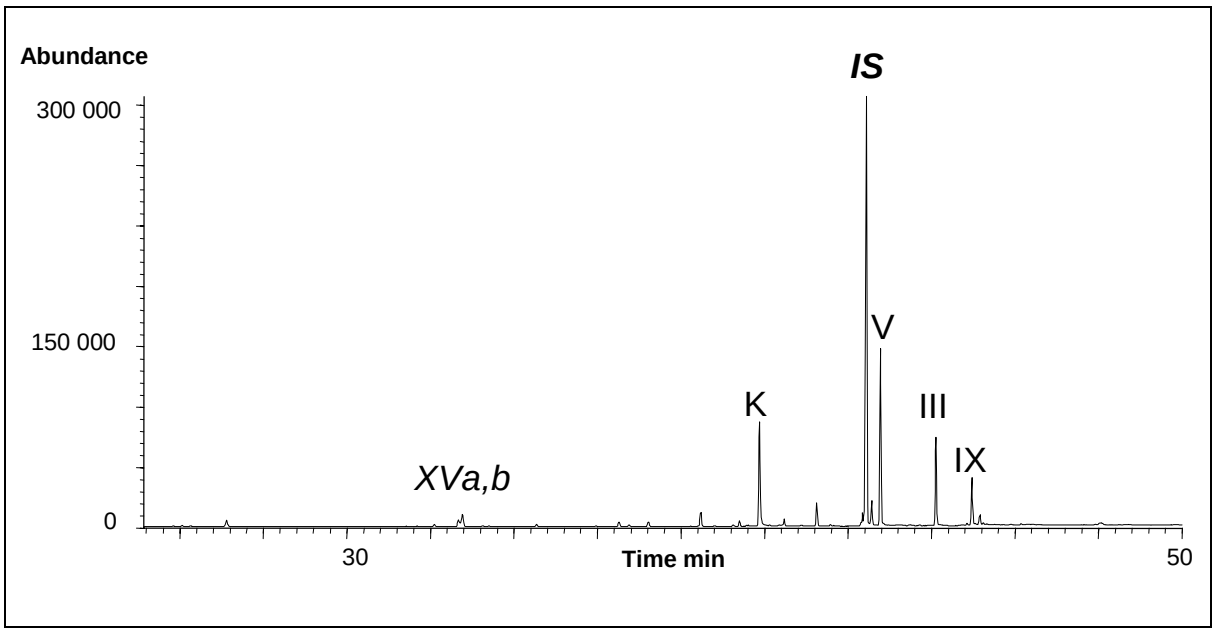


Figure 16: GC/MS TIC of reference substances at a conc. of 100 ng each: two isomers of 6-phenyl-5-hexene-2,4-dione TMS (XVa,b); kavain (K); d5-diazepam TMS (IS); 12-hydroxy-7,8-dihydrokavain TMS (V); 12-hydroxy-kavain TMS (III) and 12-hydroxy-5,6-dehydrokavain TMS (IX)

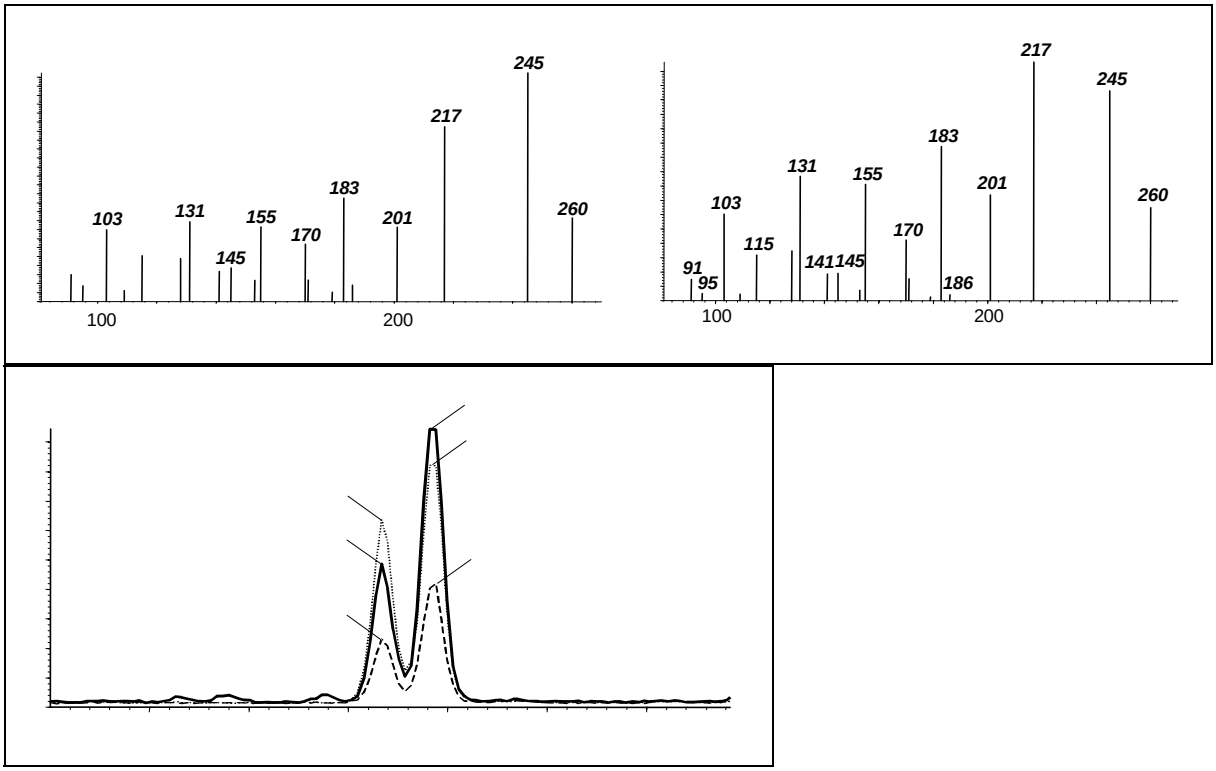


Figure 17: GC/MS EI mass spectra of the two isomers of 6-phenyl-5-hexene-2,4-dione (XV-TMS)

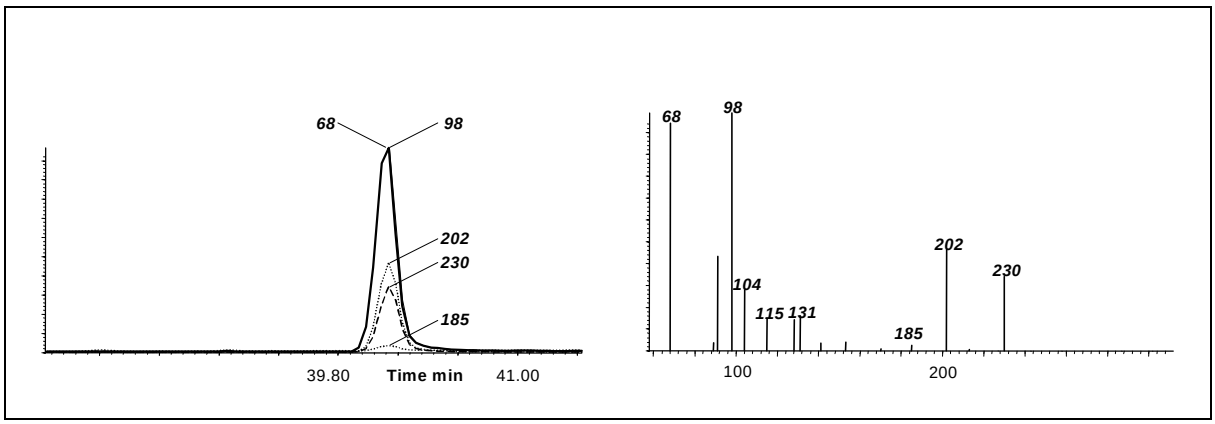


Figure 18: GC/MS EI mass spectra of D,L-kavain (K)

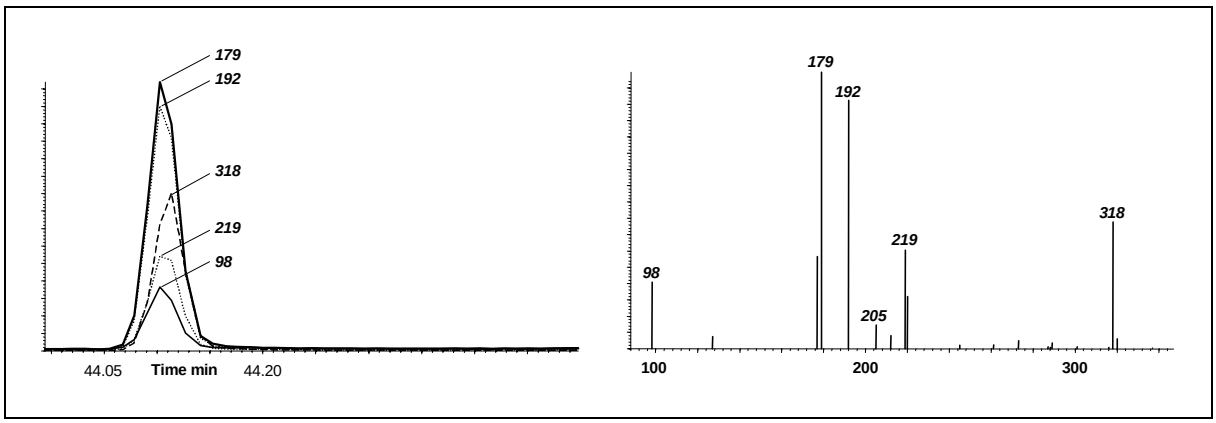


Figure 19: GC/MS EI mass spectra of 12-hydroxy-kavain (III-TMS)

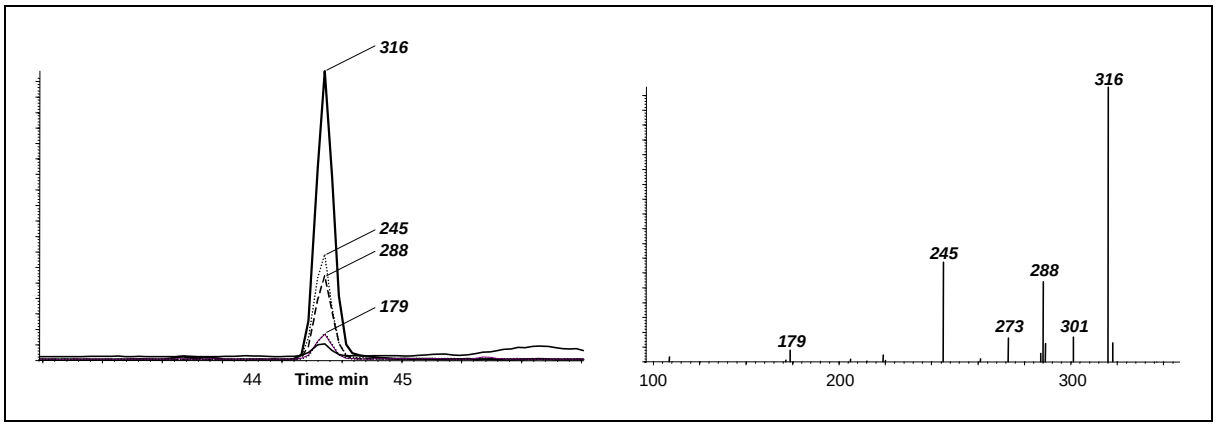


Figure 20: GC/MS EI mass spectra of 12-hydroxy-5,6-dehydrokavain (IX)-TMS

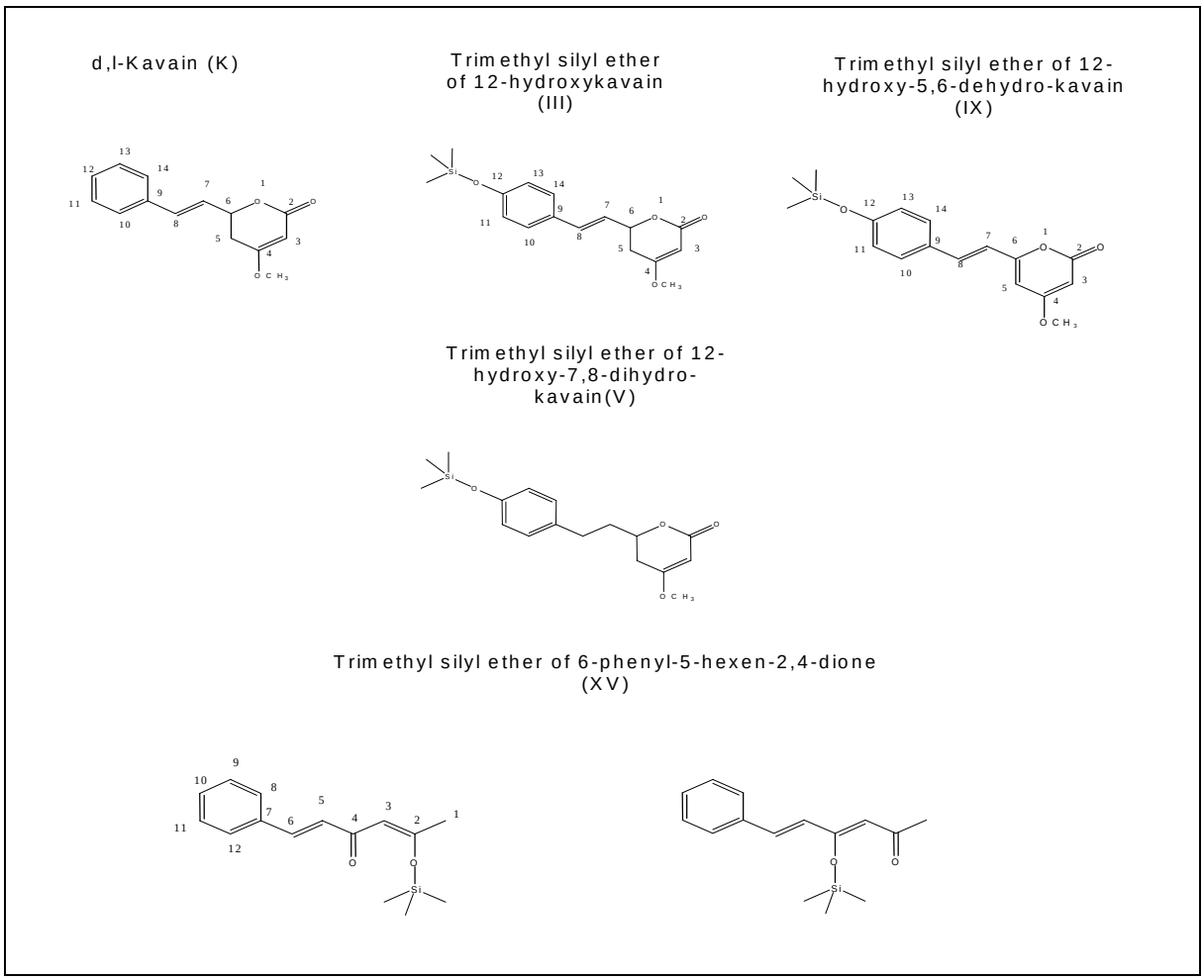


Figure 21: Chemical structures of kavain and some silylated derivatives

2.8.1.1 GC/MS Method validation

Peak identification: retention time and mass spectra of reference substances kavain, III, V, IX and XV were used to identify the peaks in the GC-chromatograms. Further metabolites were only identified by the mass spectra data from the literature (page 28, chapter 2.6.2).

Method validation was carried out as follow: the linearity for kavain and 3 of its metabolites 12-hydroxykavain (III), 12-hydroxy-7,8-dihydrokavain (V) and 12-hydroxy-5,6-dehydrokavain (IX) was proven by preparing five duplicate calibrator samples (serum extracts) at concentrations of 20, 200, 1000, 2000 and 5000 ng/ml. Linear regression was obtained by plotting the peak area ratios of substance and internal standard (d5-diazepam) versus the concentration of the substance (Figure 22).

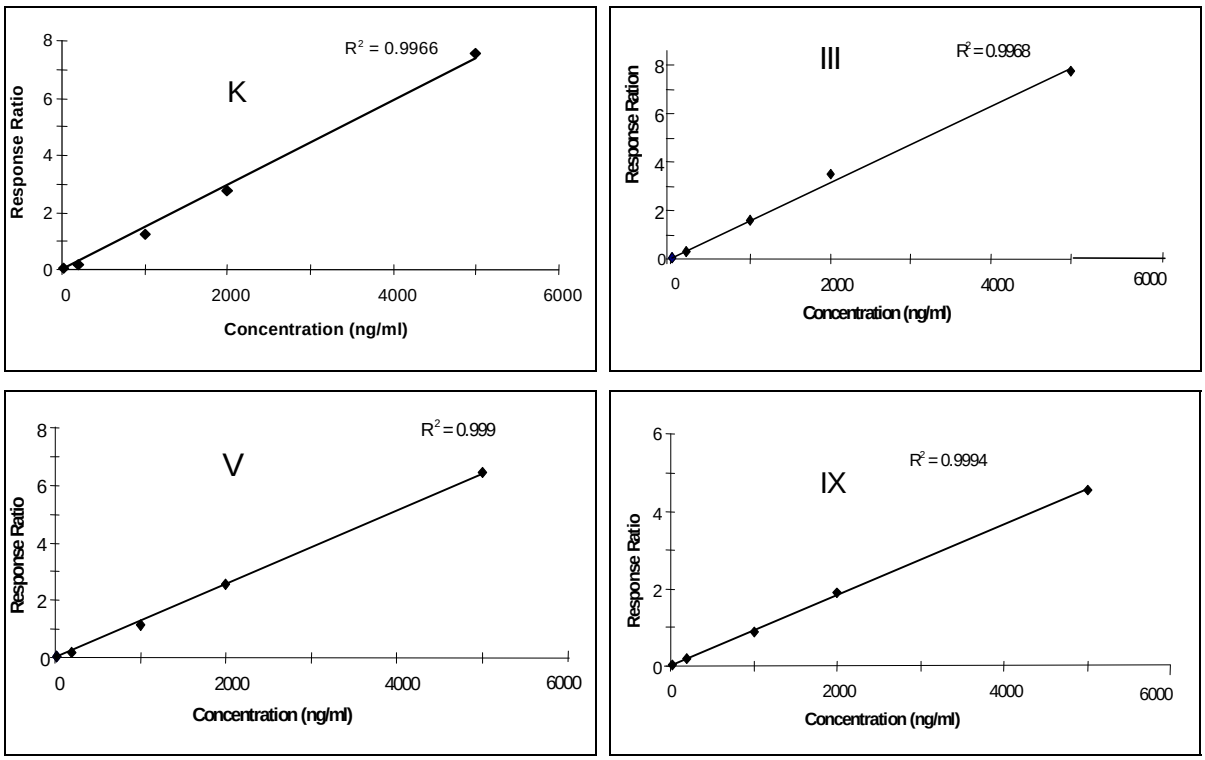


Figure 22: GC/MS method calibration curves of kavain (K), 12-hydroxykavain (III), 12-hydroxy-7,8-dihydrokavain (V) and 12-hydroxy-5,6-dehydrokavain (IX) using the GC/MS method (R2= linear regression equation)

The repeatability (within-day precision) and reproducibility (between-days precision) were carried out at a concentration of 500 ng/ml human serum as shown in table 1 and table 2.

For quantification the calibration curves (Figure 22) were used. The coefficient of variation (C.V.) ranged from 5.1 to 7.2 % for kavain, 17.5 to 19.9 % for 12-hydroxykavain, 2.4 to 4.2 % for 12-hydroxy-7,8-dihydrokavain and 1.6 to 3.5 % for 12-hydroxy-5,6-dehydrokavain. The C.V. values were acceptable for kavain and its tested metabolites (< 10 %) except for 12-hydroxykavain which showed a high C.V. value (>15 %). The GC/MS repeatability test was optimal. The accuracy test was not convenient. The high variety in recovery values observed could be due to the absence of the deuterated internal standard for kavain and its 3 metabolites and/or the derivatisation procedure. Therefore the GC/MS method can not be recommended for quantitative analyses.

Table 1: Within day precision of the GC/MS method (n = 4)

Substance	Amount added ng/ml	Mean amount ng/ml ± SD	CV %
d,l-Kavain (K)	500	465 ± 34	7.3
12-Hydroxy-7,8-dihydrokavain (V)	500	647 ± 27	4.2
12-Hydroxykavain (III)	500	309 ± 61	19.9
12-Hydroxy-5,6-dehydrokavain (IX)	500	699 ± 25	3.5

Table 2: Between-days precision of the GC/MS method (n = 4)

Substance	Amount added ng/ml	Mean amount ng/ml ± SD	CV %
d,l-Kavain (K)	500	378 ± 19	5.1
12-Hydroxy-7,8-dihydrokavain (V)	500	482 ± 12	2.4
12-Hydroxykavain (III)	500	255 ± 45	17.5
12-Hydroxy-5,6-dehydrokavain (IX)	500	604 ± 10	1.6

2.8.2 HPLC-DAD method

Peak identification was based on the on retention times and UV spectrum of kavain and 3 of its known metabolites. For the quantitation analyses, brotizolam was used as internal standard. The relative retention times kavain, metabolites and the internal standard were measured as follow: kavain (K) *RRT*=0.9 min, 12-hydroxykavain (III) *RRT*=0.43, 12-hydroxy-7,8-dihydrokavain (V) *RRT*=0.41 min and 12-hydroxy-5,6-dehydrokavain (IX) *RRT*=0.55 min. The UV spectra of kavain and its three metabolites as reference substances were measured by HPLC-DAD (Figure 23).

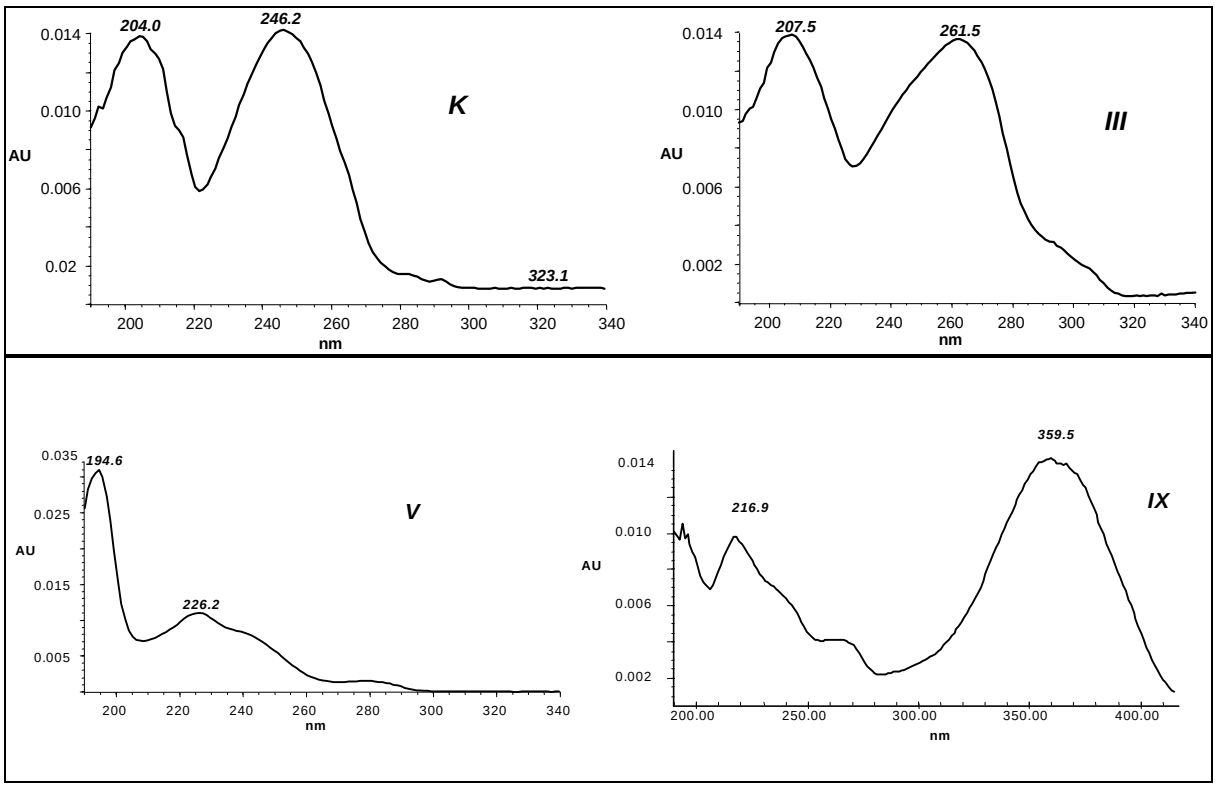


Figure 23: UV spectra of kavain and 3 of its metabolites

2.8.2.1 HPLC-DAD method validation

The linearity tests for kavain and 3 of its metabolites using their maximal absorption wavelength (246 nm for kavain, 262 nm for 12-hydroxykavain, 226 nm for 12-hydroxy-7,8-dihydrokavain and 12-hydroxy-5,6-dehydrokavain) were investigated. Due to the blood concentrations, the calibrators ranged between 5 to 100 ng/ml for kavain and between 50 to 600 ng/ml for its metabolites (Figure 24). The method was linear in these ranges.

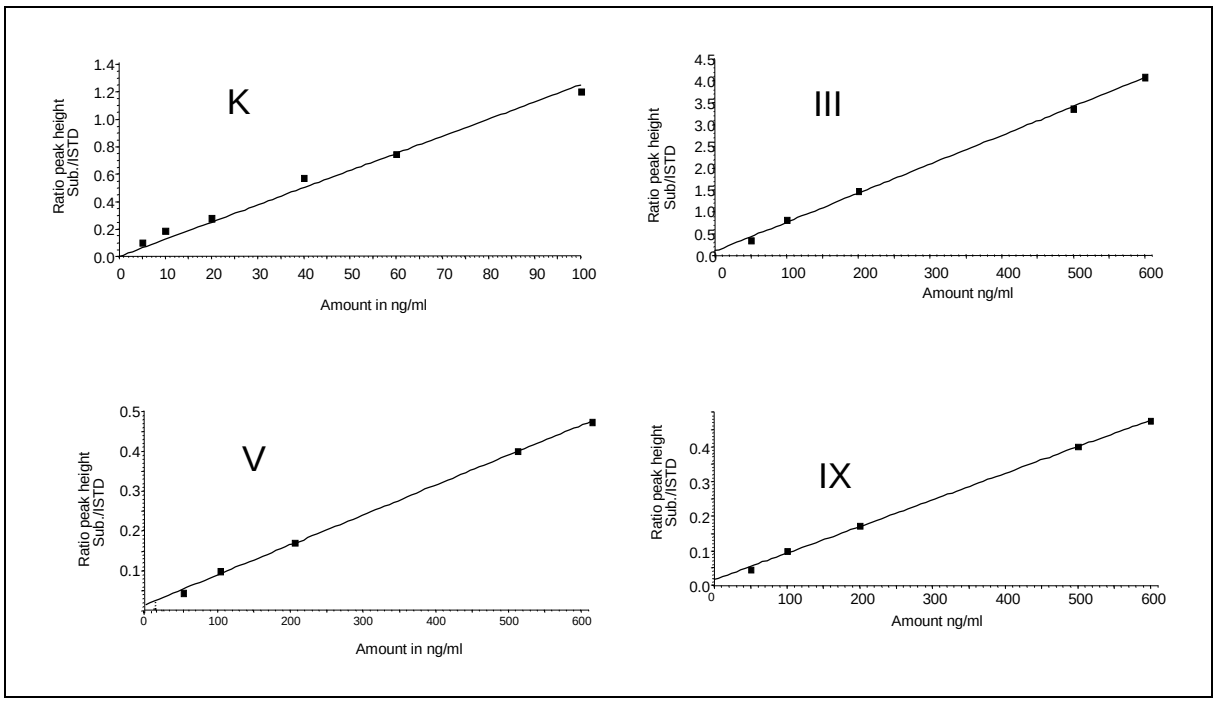


Figure 24: HPLC-DAD method: calibration curves of kavain and its metabolites in serum: kavain (K) 5-100 ng/ml, 12-hydroxykavain (III) 50-600 ng/ml, 12-hydroxy-7,8-dihydrokavain (V) 50-600 ng/ml and 12-hydroxy-5,6-dehydrokavain (IX) 50-600 ng/ml

The HPLC method was validated using the Valistat© program for the determination of kavain and its metabolites 12-hydroxykavain (III), 12-hydroxy-7,8-dihydrokavain (V) and 12-hydroxy-5,6-dehydrokavain (IX) in blood, serum and urine. The limit of detection and the limit of quantification were measured only for kavain, being 3.75 and 10.9 ng/ml respectively. The repeatability (within-day precision) and reproducibility (between-days precision) were investigated for kavain 12-hydroxykavain as shown in table 3 and table 4.

The coefficient of variation (CV) ranged from 1.17 to 7.69 % for kavain and 0.96 to 2.2 % for 12-hydroxykavain. Mean recoveries of serum extracts ranged between 91 and 97 %.

© Valistat: Programm zur statistischen Auswertung der Validierungsdaten nach den Richtlinien der GTFCh 2002 Heidelberg/Walldorf

Table 3: Intra-day precision of the HPLC-DAD method (n = 6)

Substance	Amount added ng/ml	Mean detected amount ng/ml	SD	CV in %	Mean recovery %
d,l-Kavain	5	4.9	0.37	7.69	97
	40	36.3	0.48	1.31	91
p-Hydroxykavain	50	48.0	0.84	1.74	96
	500	480.0	10.5	2.20	95

Table 4: Between-days precision (n = 6)

Substance	Amount added ng/ml	Mean detected amount ng/ml	SD	CV in %	Mean recovery %
d,l-Kavain	5	4.5	0.19	4.11	91
	40	38.6	0.45	1.17	96
p-Hydroxykavain	50	48.0	0.92	1.90	97
	500	483.0	4.65	0.96	94

2.8.3 Enzymatic cleavage of the glucuronide and sulfate of hydroxylated kavain metabolites

Glucuronide formation is one of the more common routes of drug metabolism [La Du, Mandel and Way 1979. Köppel and Tenczer 1991, Klinge Pharma 1995]. The conjugation with sulfate is usually quite limited, and can be readily exhausted. Thus with increasing doses of a drug, conjugation with sulfate becomes a less preponderant pathway [La Du, Mandel and Way 1979]. Therefore, the enzymatic hydrolyses of the hydroxylated kavain metabolites were investigated using ß-glucuronidase from E. coli and ß-glucuronidase and arylsulfatase from H. pomatia (page 35,chapter 2.7.6.2). The usage of the E. coli enzyme was useful for the cleavage of the glucuronide derivatives and the H. pomatia enzyme were suitable for the cleavage of glucuronides as well as the sulfates. Kavain metabolites were mostly conjugated [Köppel and Tenczer 1991 and Klinge Pharma 1995].

The glucuronide 12-hydroxykavain (III) needs a short incubation time (5 min) to maximise its cleavage using ß-glucuronidase from E. coli. Incubation at 45 °C with aryl sulfatase from H. pomatia showed, that the sulfate conjugate was still not completely deconjugated after 24 h (Figure 25).

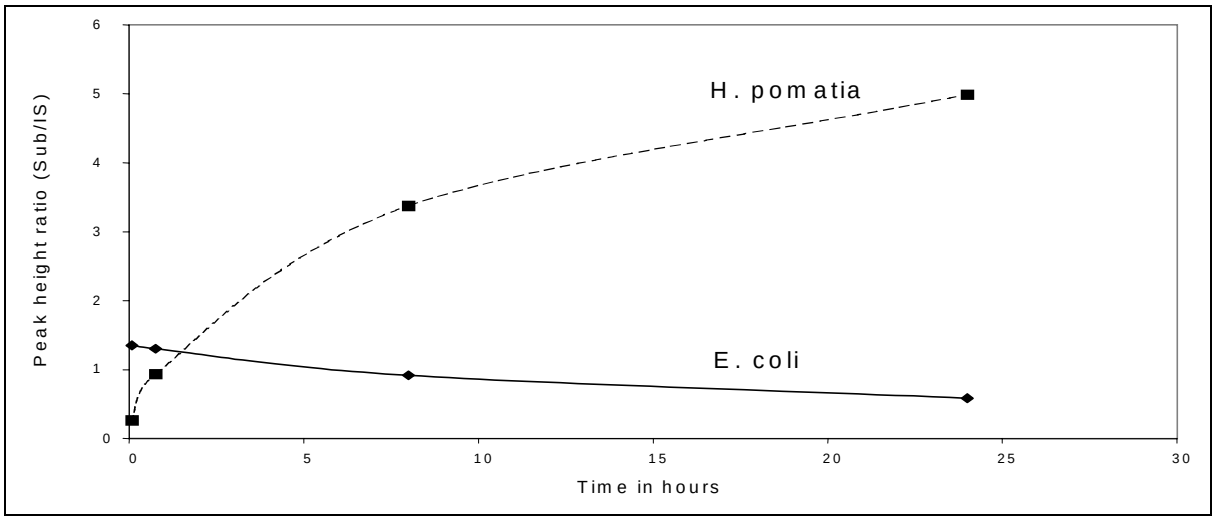


Figure 25: Time in hours needed for the cleavage of glucuronide and sulfate conjugates of 12-Hydroxykavain (III) in 24 h urine sample after kavain oral uptake (800 mg); incubation time range between 5 min and 24 h. Urine samples were analysed by HPLC-DAD

The enzymatic hydrolysis of glucuronide conjugates of 12-hydroxy-7,8-dihydrokavain (V) using ß-glucuronidase from E. coli needs only a short incubation time (5 min) to reach the maximum. On the other hand the cleavage of the sulfate conjugates using arylsulfatase from H. pomatia needs at least 8 hours at 45 °C (Figure 26).

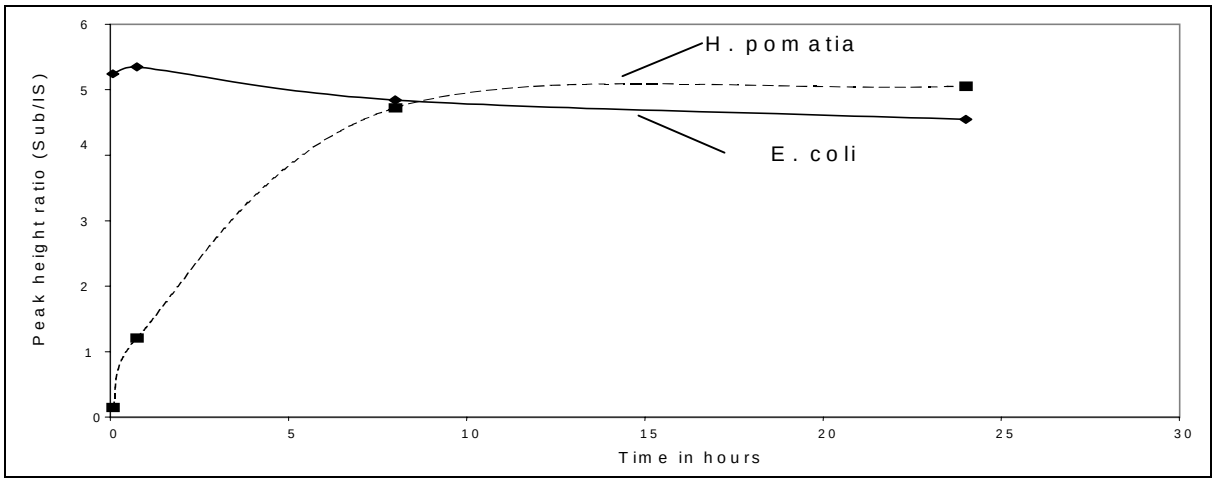


Figure 26: Time in hours needed for the cleavage of glucuronide and sulfate conjugates of 12-Hydroxy-7,8-dihydrokavain (V) in 24 h urine sample after kavain oral uptake (800 mg); incubation time range between 5 min and 24 h. Urine samples were analysed by HPLC-DAD

The enzymatic hydrolyses of the unidentified metabolite (XVIII) in the urine samples showed that this metabolite is excreted in urine mainly as glucuronide. It has a characteristic UV spectrum which is related to kavain. The enzymatic hydrolyses using ß-glucuronidase from E. coli only needs a short incubation time 5 min (Figure 27).

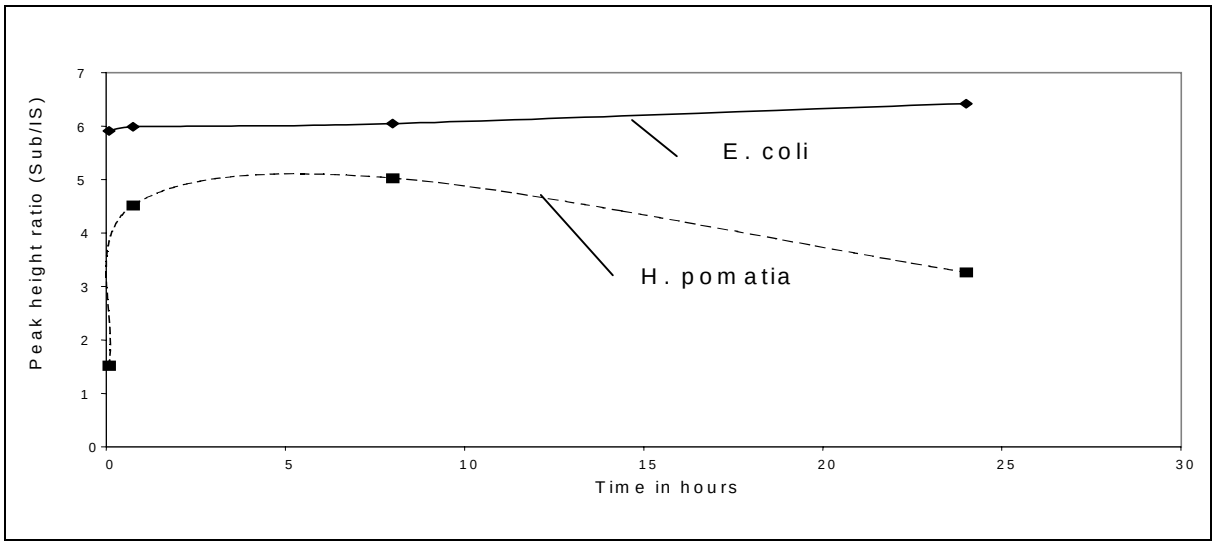


Figure 27: Time in hours needed for the cleavage of glucuronide and sulfate conjugates of the unidentified metabolite (XVIII) in 24 h urine sample after kavain oral uptake (800 mg); incubation time range between 5 min and 24 h. Urine samples were analysed by HPLC-DAD

3 Results

3.1 Kavain metabolism by means of Hep-G2 cell cultures

GC/MS analyses of the Hep-G2 cell cultures after incubation with D,L-kavain revealed unchanged kavain and fourteen metabolites. Four metabolites were detected without derivatisation VI, XI, XIII and XIV (Figure 28 and Table 5). One metabolite (kava acid methyl ester X) could only be identified after methylation (Figure 29 and Table 6). Nine metabolites I, III, IV, V, XV, XVI, XVII, XVIII and XIX were detected after silylation (Figure 31 and Table 7). The position of the hydroxy group of (I) is still unknown. It was identified according to its mass fragmentation and the data from the literature [Rasmussen et al. 1979]. The relative amount of metabolite (V) significantly increased after the enzymatic hydrolyses using ß-glucuronidase from E. coli. This metabolite IVa is considered to be a glucuronide and / or sulfate conjugate of IV. 12 of 17 previously published urinary metabolites could also be detected in Hep-G2 cells.

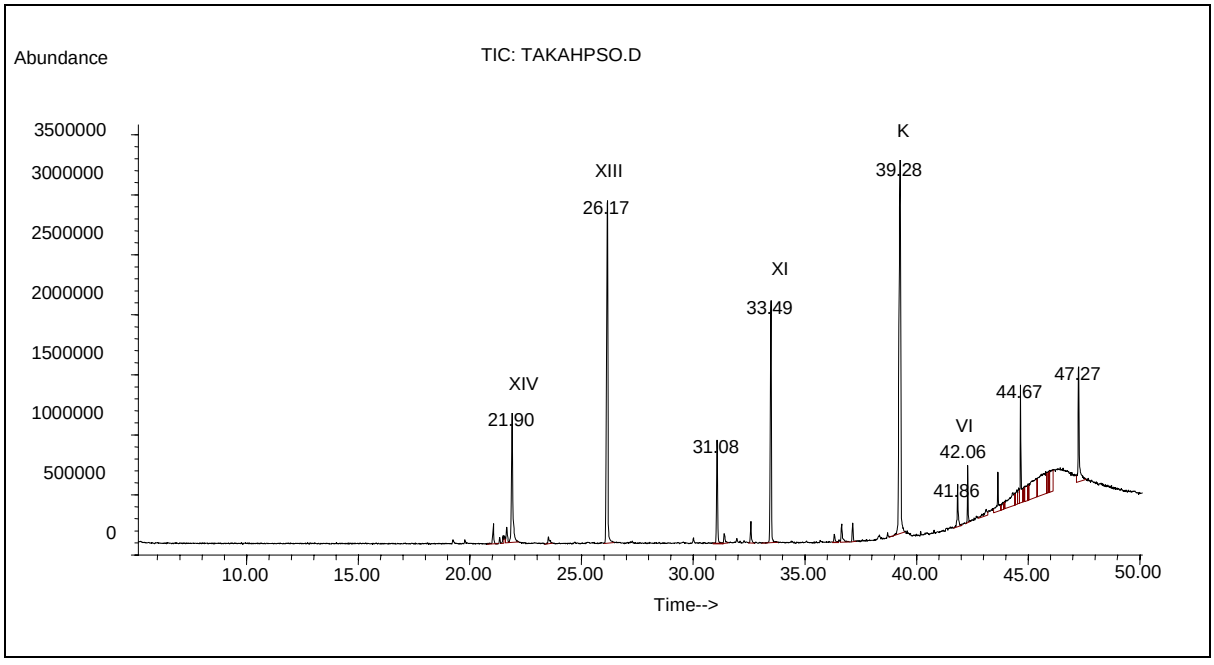


Figure 28: GC/MS chromatogram of SIM mode: Hep-G2 cell after incubation with D,L-kavain: total ion chromatograph of the cell culture extract (underivatised): kavain (K), cinnamyl-acetone (XI), 4-oxy-cinnamyl-acetone (XIII), 6-phenyl-3-hexene-2-one (XIV)

Table 5: None derivatised extract of the Hep-G2-cell after incubation with kavain

Substance name	RT min	Prominent Fragments (m/z)
Kavain (K)	39.28	230 $[M]^+$ (30), 202 (45), 131 (25), 115 (23), (29), 98 (100), 91 (40), 68 (95)
5,6-Dehydrokavain (VI)	42.06	228 $[M]^+$ (100), 157 (65), 200 (45), 185 (35), 103 (37), 115 (20), 129 (38), 78 (43), 69 (60)
Cinnamyl-acetone (XI)	33.49	189 $[M]^+$,104 (100), 121 (90), 77 (50), 133 (10), 91 (12), 107 (7)
4-Oxy-cinnamyl-acetone (XIII)	26.17	188 $[M]^+$ (100), 127 (90), 77 (50), 115 (40), 121 (37), 91 (20), 173 (15), 131 (2), 141 (3), 86, 98, 104
6-Phenyl-3-hexen-2-one (XIV)	21.90	174 $[M]^+$, 129 (36), 117 (60), 115 (50), 91 (100), 104 (40), 77 (25)

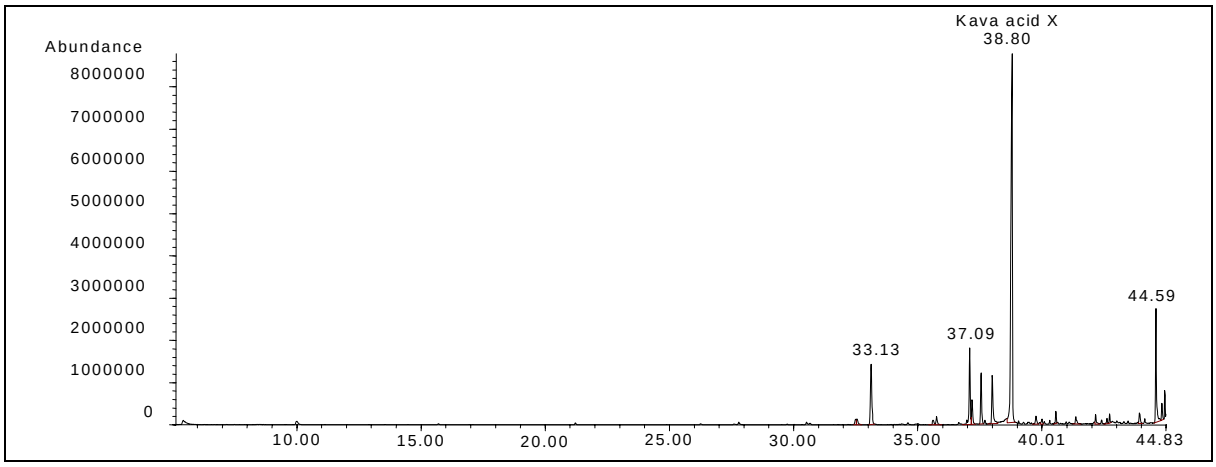


Figure 29: GC/MS chromatogram of SIM mode: Hep-G2 cell after incubation with D,L-kavain: total ion chromatogram of the cell culture extract (after derivatisation by methylation)

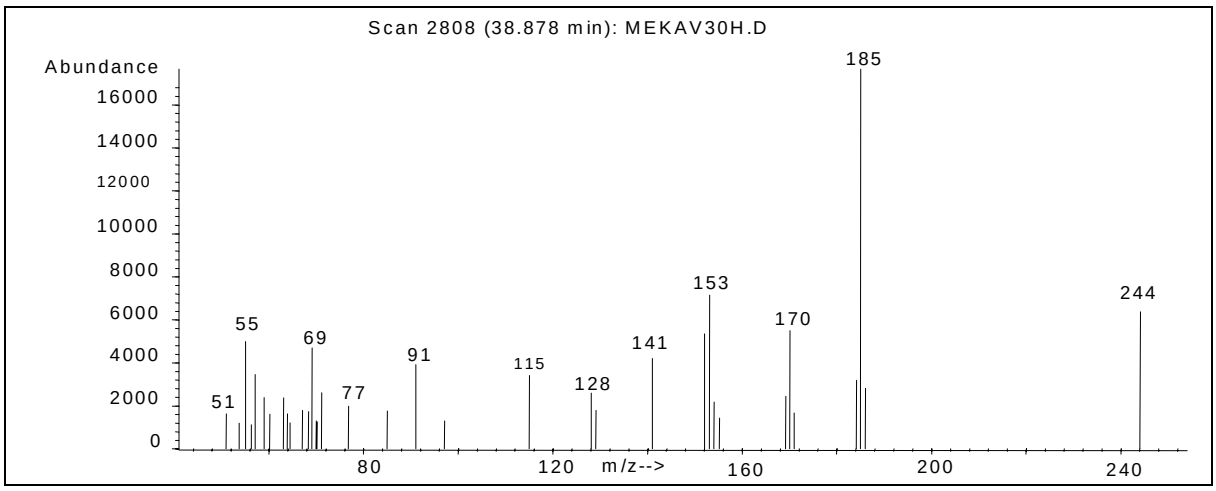


Figure 30: GC/MS EI mass spectra of kava acid methyl ester (X)

Table 6: Methylated metabolite of D,L-kavain after incubation with the Hep-G2-cell

Substance name	RT min	Prominent Fragments (m/z)
Kava acid methyl ester (X)	38.80	244 $[M]^+$ (40), 185 (100), 153 (50), 170 (45), 141 (40), 115 (38), 128 (35), 69 (30)

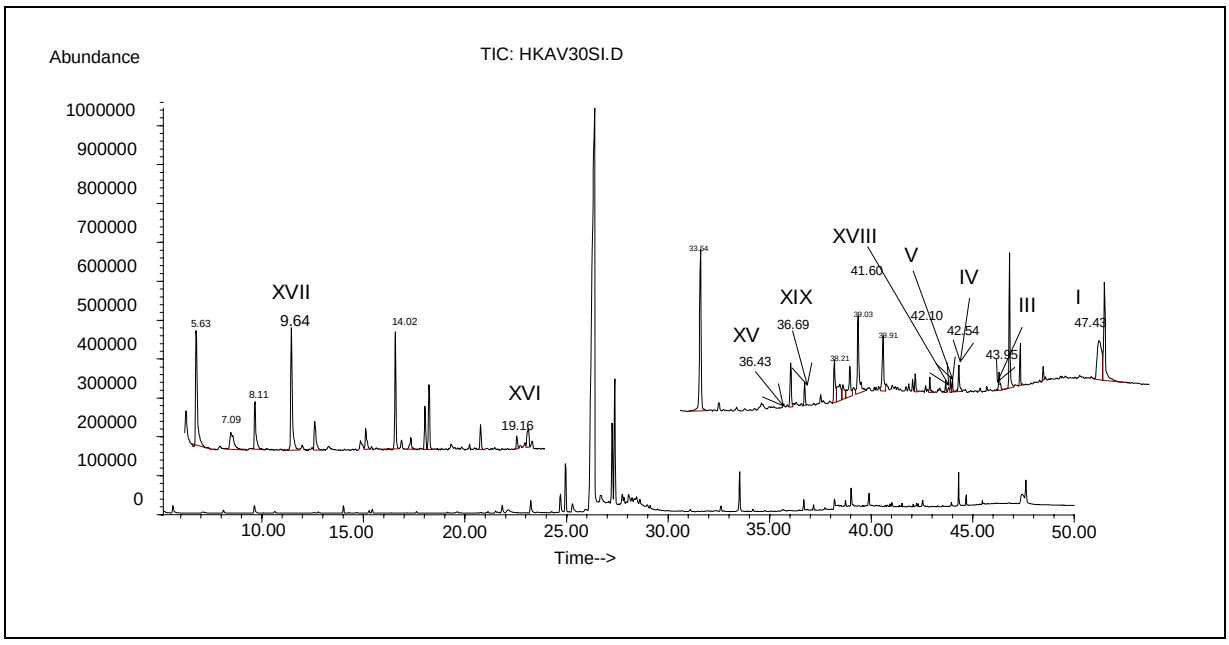


Figure 31: GC/MS chromatogram in SIM mode: Hep-G2 cell after incubation with D,L-kavain: total ion chromatograph of the cell culture extract (after derivatisation by silylation): hydroxykavain (I), 12-hydroxykavain (III), 11-hydroxy-7,8-dihydrokavain (IV), 12-hydroxy-7,8-dihydrokavain (V), 6-phenyl-5-hexene-2,4-dion (XV), p-hydroxybenzoic acid (XVI), unidentified metabolite (XVIIII)

Table 7: Kavain and its metabolites detected in Hep-G2 cells culture after derivatisation by silylation

Substance	RT min	Prominent Fragments (m/z)
Hydroxykavain (I)	47,43	318 $[M]^+$ (12), 273 (32), 243 (15), 228 (26), 189 (16), 173 (38), 107 (100), 91 (98), 69 (80),133 (60), 121 (58)
12-Hydroxy-kavain (III)	43.95	318 $[M]^+$ (50), 179 (100), 192 (98), 219 (45), 205 (25), 98 (46)
11-Hydroxy-7,8-dihydro-kavain (IV)	42.54	320 $[M]^+$ (40),305 (18), 179 (100), 179, 192 (10), 127)30), 205 (60)
12-Hydroxy-7,8-dihydro-kavain (V)	42.10	320 $[M]^+$ (50), 179 (100), 192 (10), 127 (30), 205 (60)
6-Phenyl-5-hexen-2,4-dione (XV)	36.43	260 $[M]^+$ (34), 245 (98), 217 (100), 183 (76), 103 (40), 115 (30), 170 (32), 155 (60), 131 (65)
p-Hydroxy-benzoic acid (XVI)	19.16	224 (100), 210 (30), 209 (92), 193 (56), 179 (12), 177 (52), 149 (35), 135 (66)
Unidentified urine metabolite (XVII)	9.64	323 $[M]^+$ (80), 193 (100), 179 (60), 205 (55), 224 (20), 293 (22), 135 (10), 115 (7)
Unidentified metabolite (XVIII)	41.60	302 (35), 287 (25), 258 (16), 243 (36), 185 (100), 170 (16), 153 (38), 141 (30), 128 (25), 115 (28), 89 (29)
4-Hydroxy-6-hydroxy-phenyl-5-hexen-2-one (XIX)	36.69	350 $[M]^+$ (7), 335 (10), 294 (24), 293 (100), 219 (12), 203 (16), 193 (18), 179 (42), 143 (21), 115 (28)

The GC/MS analyses of the Hep-G2 cells extract and the human urine showed that the kavain metabolite XIII (4-Oxycinnamyl-acetone) is detected only in cell culture extract (Figure 32).

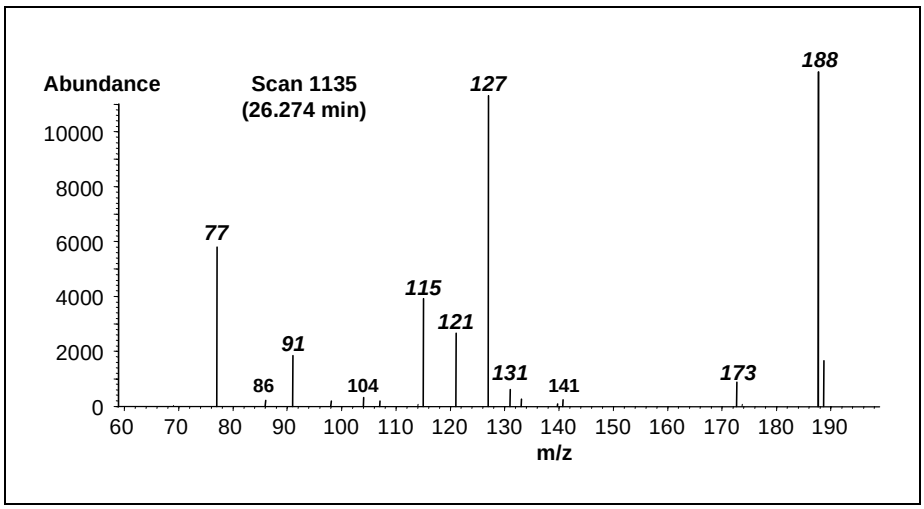


Figure 32: GC/MS EI mass spectrum of 4-oxy-cinnamyl-acetone (XIII) detected only in cell culture extract.

3.2 Metabolism of kavain in human

3.2.1 Determination of kavain and its metabolites in urine and blood samples by GC/MS

For the metabolism study of kavain by human, we started our experimental studies by means of an application of D,L-kavain orally at a dose of 200 mg single dose. The selection of this dose was based on the product information from Klinge Pharma. The propose was to observe how kavain metabolised by human and to compare the results with the previous work [Klinge Pharma 1995]. The blood samples were collected 45 min,1.5 h, 3 h, 6 h, 12 h and 24 h after the oral uptake. The first sample showed only the presence of kavain (K) and 5,6-dehydrokavain (VI). The other metabolites (12-hydroxykavain (III), 12-hydroxy-7,8-dihydrokavain (V) and 12-hydroxy-5,6-dehydrokavain) were not detected. 12-Hydroxykavain (free form) was detected after 1,5 h up to 12 h but not after 24 h. 12-Hydroxy-7,8-dihydrokavain was not detected in blood and serum. The urine samples were collected 2, 5, 12, 24 h after the oral dose. All samples were stored at - 20 °C till analyses. Urine samples were analysed before and after enzymatic hydrolyses using ß-glucuronidase from E. coli (page 34, chapter 2.7.5.3). Kavain was not detected in urine but the metabolite 12-hydroxykavain was extracted in urine in its free and conjugated form (glucuroinde and sulfate forms). No kinetic investigations were done with this oral dose.

3.2.1.1 Determination of kavain and its metabolites in urine samples

This trial of the self medication study on kavain metabolism was carried out after application of 800 mg D,L-kavain as single oral dose. Urine samples were collected before kavain uptake for the blank studies, followed by the collection of urine after 2, 5, 9, 12 and 24 h. From the whole urine samples a 24 h urine sample was collected. All samples were stored at - 20 °C till analyses. Urine samples were analysed before and after enzymatic hydrolyses using ß-glucuronidase from E. coli (page 34, chapter 2.7.5.3). Determination of kavain and its hydroxylated metabolites was carried out by GC/MS and d5-diazepam as internal standard. The analyses were approved before and after derivatisation (silylation or methylation) of the samples in order to detect the hydroxylated metabolites of kavain (page 33, chapter 2.7.5.).

Unchanged kavain could not be detected in urine up to 24 h. Only 3 metabolites were detected without derivatisation. The most important metabolite was 5,6-dehydrokavain (VI, *RT*: 40.5 min.) which was identified by its mass spectrum with its characteristic fragments m/z: 228 $[M]^+$ (100), 157 (65), 200 (45), 185 (35), 103 (37), 115 (20), 129 (38), 78 (43), 69 (60) (Figure 33) [Köppel and Tenczer 1991]

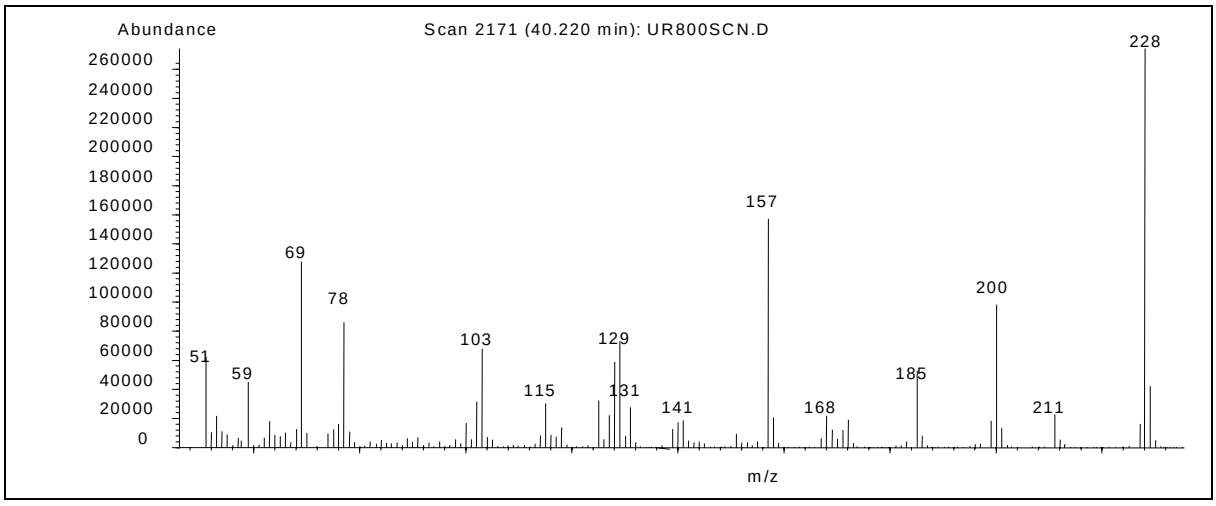


Figure 33: GC/MS (TIC and EI mass spectra) of 5,6-dehydro-kavain (VI) in urine without derivatisation after a single oral dose of 800 mg D,L-kavain

Due to the lacking of 5,6-dehydrokavain as reference substance, we have compared our mass fragments to those published by Köppel and Tenczer 1991, both which were identical. This metabolite showed high concentrations during the first 2 h, which then gradually decreased up to 24 h (Table 8). The two metabolites detected without derivatisation were cinnamyl-acetone (XI, *RT:* 33.4 min) which was identified by its

characteristic fragments m/z 189 $[M]^+$,104 (100), 121 (90), 77 (50), 133 (10), 91 (12), 107 (7) and 6-phenyl-3-hexene-2-one (XIV, *RT:* 21.9 min) with its characteristic fragments m/z 174 $[M]^+$, 129 (100), 117 (60), 115 (55), 91 (48), 143 (40), 85 (25), 158 (20) (Table 9).

The non conjugated and the conjugated forms of the hydroxylated kavain metabolites were identified after TMS silylation. The main urinary metabolites were 12-hydroxy-kavain (III), 12-hydroxy-7,8-dihydrokavain (V), 12-hydroxy-5,6-dehydrokavain (IX) and 6-phenyl-5-hexene-2,4-dione (XVa,b) (Figure 34).

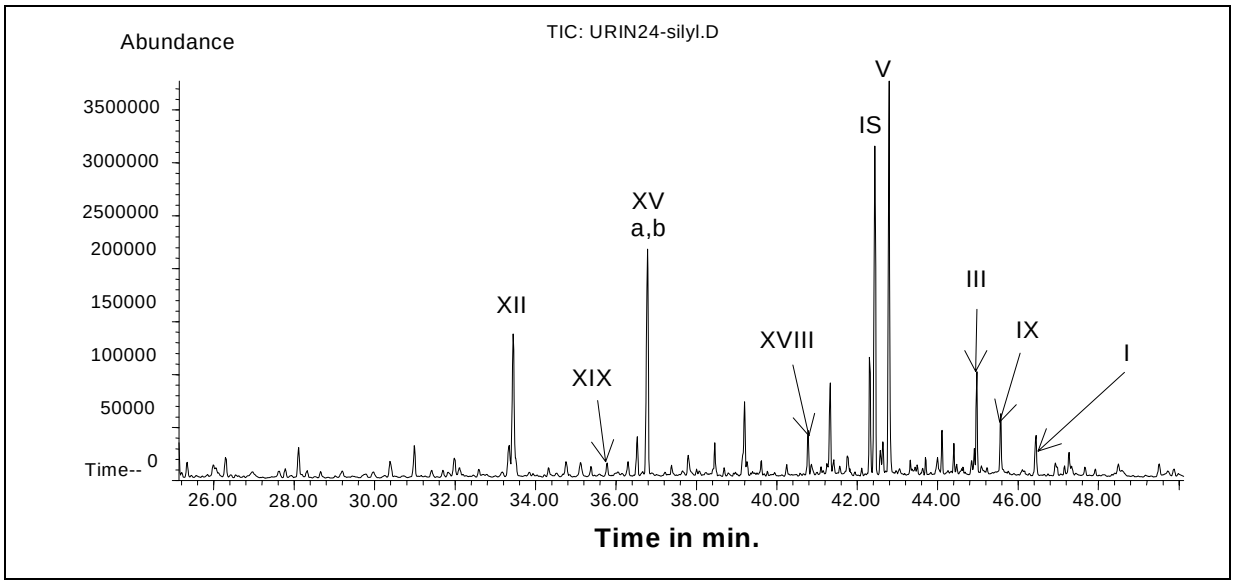


Figure 34: GC/MS (TIC) of the silylated urine extract after a single oral dose of 800 mg D,L-kavain:The following metabolites could be identified: two isomers of 6-phenyl-5-hexene-2,4-dione (XV a,b), hydroxykavain (I), 12-hydroxy-7,8-dihydrokavain (V), 12-hydroxy-kavain (III),unidentified metabolite (XVIII) and 12-hydroxy-5,6-dehydrokavain (IX), 4-hydroxy-cinnamyl-acetone (XII), 4-hydroxy-6-hydroxyphenyl-5-hexen-2-one (XIX). IS= the internal standard (*d5*-diazepam)

The metabolite 12-hydroxy-7,8-dihydrokavain (V) was detected in the early samples at low concentration which then significantly increased between 5 and 12 h from kavain oral up take. A rapid elimination after 12 h was observed (Table 8). The metabolite 12-hydroxykavain (III) was found to be at high concentrations from the beginning of the experiment with constant high concentrations up to 12 h (Table 8). The metabolite 12-hydroxy-5,6-dehydrokavain (IX) was present in the first urine at high concentrations with a slow elimination rate. The unidentified metabolite (XVIII)

was detected after derivatisation by silylation, its mass spectrum fragments could not explain its origin (Figure 35). The diagram showed the molecular ion with m/z 302 which could be formed after desmethylation of 12-hydroxy-5,6-dehydrokavain (IX) at the position 4 of the lactone ring, or the metabolite 5,6-dehydrokavain is first hydroxylated at the phenyl ring and then desmethylated at the C-4 of the lactone ring.

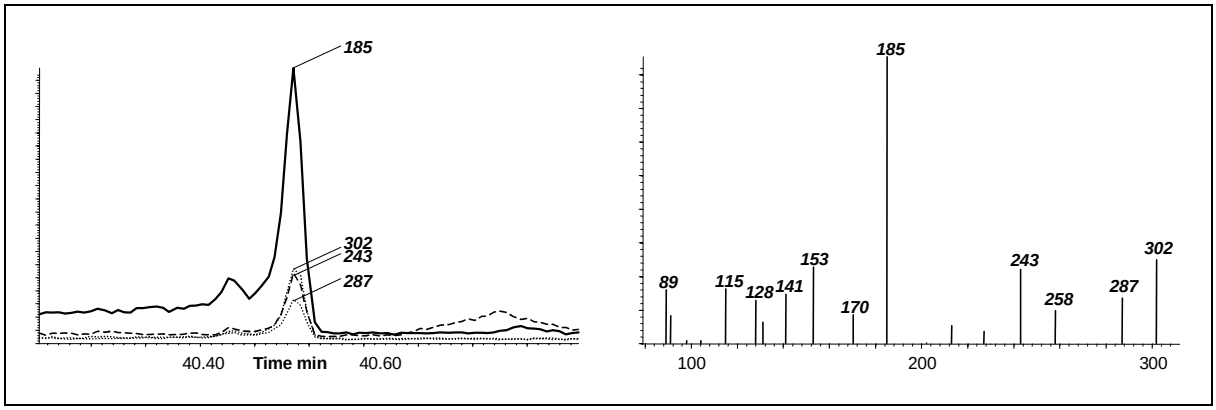


Figure 35: GC/MS (EI mass spectra) of unidentified metabolite (XVIII) in urine after a single oral dose of 800 mg D,L-kavain (after derivatisation by silylation)

The metabolite 6-phenyl-5-hexene-2,4-dione (XV) was found in all the urine samples but its signal was disturbed and overlapped with endogenous compounds. This overlap makes it difficult to quantify it (Table 8).

Table 8: Urinary metabolites (total amounts of their free and glucuronide form collected from 2 to 24 h after a single oral dose (800 mg) of D,L-kavain using GC/MS method

	Concentration in ng/ml				
Substance	**0-2 h**	**2-5 h**	**5-9 h**	**9-12 h**	**12-24 h**
D,L-kavain	not detected				
5,6-Dehydro-kavain	detected but not quantified				
12-Hydroxy-7,8-dihydrokavain	97	105	3630	> 5000	190
12-Hydroxykavain	> 5000*	> 5000*	> 5000*	> 5000*	610
12-Hydroxy-5,6-dehydrokavain	4300	> 5000*	4160	2450	1140
6-Phenyl-5-hexen-2,4-dione	detected but not quantified				

*concentration higher than the highest calibrator

Further kavain metabolites found in the urine samples detected with and without derivatisation are listed together with their retention times and characteristics mass fragments. Three metabolites (VI, XI and XIV) were detected without derivatisation (Table 9, 10 and 11).

Table 9: None derivatised urinary metabolites after single oral dose of D,L-kavain

Substance name	**RT min**	**Prominent Fragments (m/z)**
5,6-Dehyrokavain (VI)	40.22	228 $[M]^+$ (100), 157 (65), 200 (45), 185 (35), 103 (37), 115 (20), 129 (38), 78 (43), 69 (60)
Cinnamyl-acetone (XI)	33.4	189 $[M]^+$,104 (100), 121 (90), 77 (50), 133 (10), 91 (12), 107 (7)
6-Phenyl-3-hexene-2-one (XIV)	21.9	174 $[M]^+$, 129 (100), 117 (60), 115 (55), 91 (48), 143 (40), 85 (25), 158 (20)

Nine of the hydroxylated kavain metabolites (I, III, V, IX, XII,XV, XVI, XVIII and XIX) were successfully detected after silylation (Table 10).

Table 10: Hydroxylated kavain urinary metabolites detected by GC/MS after derivatisation by silylation

Substance	RT min	Prominent Fragments (m/z)
Hydroxykavain (I)	46.89	318 $[M]^+$ (12), 273 (32), 243 (15), 228 (26), 189 (16), 173 (38), 105 (100), 91 (98), 69 (80),133 (60), 121 (58)
12-Hydroxy-kavain (III)	44.36	318 $[M]^+$ (50), 179 (100), 192 (98), 219 (45), 205 (25), 98 (46).
12-Hydroxy-7,8-dihydro-kavain (V)	43.41	320 $[M]^+$ (50), 179 (100), 192 (10),.127 (30), 205 (60).
12-Hydroxy-5,6-dehydro-kavain (IX)	45.94	316 $[M]^+$ (100), 245 (40), 260 (12), 273 (19), 179 (7), 115 (6)
4-Hydroxy-cinnamyl-acetone (XII)	33.97	260 [M]+ (100), 245 (80), 115 (78), 205 (45), 179 (43), 105 (48), 135 (40), 128 (37), 127 (35),
6-Phenyl-5-hexen-2,4-dione (XV)	36.45	260 $[M]^+$ (34), 245 (98), 217 (100), 183 (76), 103 (40), 115 (30), 170 (32), 155 (60), 131 (65).
p-Hydroxy-benzoic acid (XVI)	19.24	224 (80), 205 (7), 193 (100), 179 (40), 177 (62), 135 (20)
Unidentified metabolite (XVIII)	40.58	302 (35), 287 (25), 258 (16), 243 (36), 185 (100), 170 (16), 153 (38), 141 (30), 128 (25), 115 (28), 89 (29)
4-Hydroxy-6-hydroxy-phenyl-5-hexen-2-one (XIX)	35.94	350 $[M]^+$ (7), 323 (10), 293 (100), 260 (12), 205 (16), 192 (8), 179 (9), 134 (15), 115 (12)

The metabolite kava acid methyl ester (X) was detected only when the urine sample was derivatised by methylation (Table 11).

Table 11. Urinary metabolite detected only after methylation

Substance name	RT min	Prominent Fragments (m/z)
Kava acid methyl ester (X)	39.28	244 $[M]^+$ (40), 185 (100), 153 (50), 170 (45), 141 (40), 115 (38), 128 (35), 69 (30)

3.2.1.2 Determination of kavain and its metabolites in blood samples

According to the first trial after application of the 200 mg single oral dose, we collected for the determination of kavain and its metabolites in a next trial 3 blood samples 0.5 h, 8 h and 24 h after a single oral dose of 800 mg of kavain. The chemical structure of kavain and some of its metabolites are shown in figure 36.

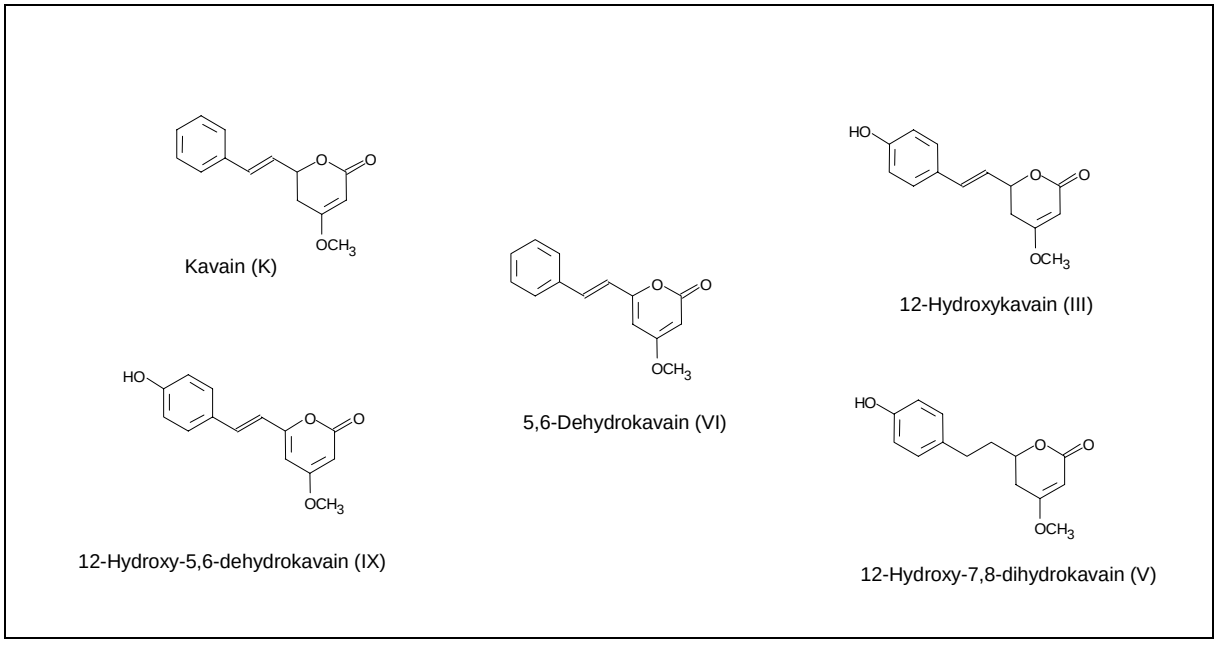


Figure 36: The structure of kavain and its metabolites

Blood samples were analysed before and after enzymatic hydrolyses and with and without silylation. Unchanged kavain was detected in the sample taken after half hour. The metabolite 12-hydroxykavain (III) was detected before and after the enzymatic hydrolyses. The metabolites 12-hydroxy-7,8-dihydrokavain (V) and 12-hydroxy-5,6-dehydrokavain (IX) were detected in the first sample and only after enzymatic treatment. The metabolite 6-phenyl-5-hexene-2,4-dione (XV) was not detected in blood (Table 12).

Table 12: Blood metabolites in their free and conjugated form during a time course of up to 24 h after a single oral dose (800 mg) of D,L-kavain using GC/MS method

	Concentration in ng/ml			
Time in hours	**0.5 h**	**0.5 h**	**8 h**	**24 h**
Substance	before enzymatic hydrolysis	after enzymatic hydrolysis	after enzymatic hydrolysis	after enzymatic hydrolysis
d,l-kavain (K)	+	+	n. d.	n. d.
5,6-Dehydro-kavain (VI)	n. d.	n. d.	n. d.	n. d.
12-Hydroxy-7,8-dihydrokavain (V)	n. d.	++	n. d.	n. d.
12-Hydroxykavain (III)	+++	++++	++	n. d.
12-Hydroxy-5,6-dehydrokavain (IX)	n. d.	++	n. d.	n. d.
6-Phenyl-5-hexen-2,4-dione (XV)	n. d.	n. d.	n. d.	n. d.

n. d.: not detected

The exposure to temperatures during derivatisation and by the injection into the GC/MS system revealed some products that seemed to be pyrolysis products e.g. opening of the lactone ring of kavain followed by decarboxylation. Even if we analysed the reference substance kavain we found a pyrolysis product (Figure 37) which is defined in the GC/MS library as kavain metabolite [Pfleger, Maurer and Weber 2000].

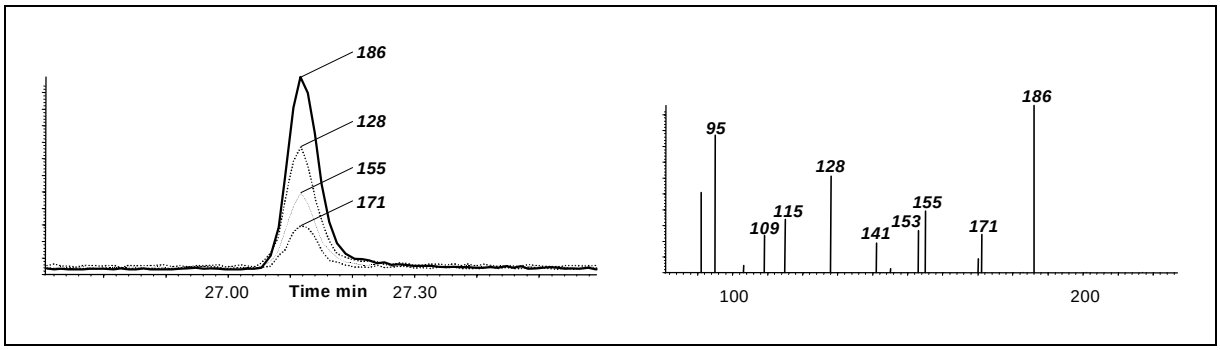


Figure 37: GC/EI mass spectrum of decarboxylated kavain (-CO_2)

3.2.2 Determination of kavain and its metabolites in urine and blood samples using HPLC-DAD

The self medication was also carried out with aim to study the D,L-kavain metabolism by human using HPLC-DAD method. All of the samples analysed before and after the enzymatic hydrolyses using the optimised procedure as described in chapter 2.8.3.

3.2.2.1 Determination of kavain and its metabolites in urine using the HPLC-DAD method

The self medication study on kavain metabolism was carried out after application of 800 mg D,L-kavain as single oral dose. Urine samples were collected before kavain uptake for the blank studies, followed by the collection of urine after 2, 4, 8, 18 and 24 h. From the whole urine samples a 24 h urine sample was collected. All samples were stored at - 20 °C till analyses. Urine samples were analysed before and after enzymatic hydrolyses using ß-glucuronidase from E. coli (see page 34, chapter 2.7.5.3).

The enzymatic hydrolysis using ß-glucuronidase from E. coli and ß-glucuronidase / aryl sulfatase from H. pomatia proved that the hydroxylated kavain metabolites are mostly excreted in their conjugated form [Köppel and Tenczer 1991, Klinge Pharma 1995]. The 24 h urine sample was the best presentation of the enzymatic conjugation effect on the hydroxylated metabolites of kavain. Concentration of the metabolite 12-hydroxykavain (III) was extremely increased after the incubation for only 5 min with E. coli (Figure 38).

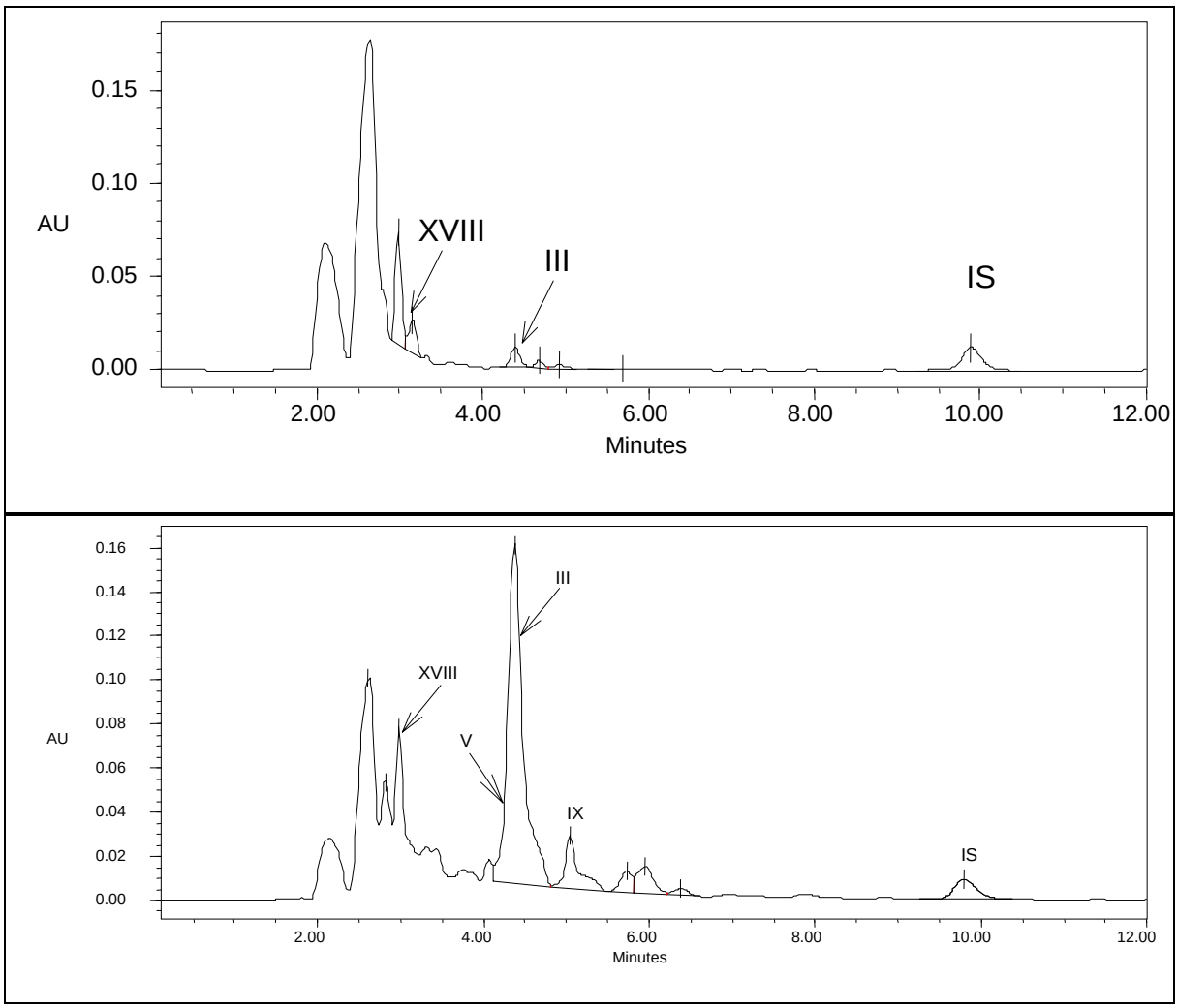


Figure 38: HPLC chromatogram at 262 nm: urine sample 8 h after the oral uptake of 800 mg kavain:12-hydroxykavain (III), 12-hydroxy-7,8-dihydrokavain (V), 12-hydroxy-5,6-dehydrokavain (IX) and the unidentified metabolite (XVIII) (top without and bottom with incubation with E. coli)

The very high signal of 12-hydroxykavain (III) overlapped the signal of 12-hydroxy-7,8-dihydrokavain (V) as shown in figure 38. For the quantitative analyses of kavain and its 3 metabolites, specific and selected wavelengths were used as described in chapter 2.6, page 24.

Table 13: Urinary metabolites (free, glucuronide and the whole glucuronide and sulfate) after a single oral dose (800 mg) of D,L-kavain using HPLC-DAD (n. d.: not detected, sulf: sulfate, glu: glucuronide)

	Concentration in ng/ml				
Substance	**0-2 h**	**2-4 h**	**4-8 h**	**8-18 h**	**18-24 h**
D,L-kavain	n. d.	n. d.	n. d.	n. d.	n. d.
12-Hydroxykavain (III) free	130	225	167	79	60
12-Hydroxykavain (III) glu	2047	3105	3500	3950	2626
12-Hydroxykavain (III) glu + sulf	8505	10900	15379	14217	7923
12-Hydroxy-7,8-dihydrokavain (V) free	n.d.				
12-Hydroxy-7,8-dihydrokavain (V) glu	n.d.	n.d.	3265	6680	4316
12-Hydroxy-7,8-dihydrokavain (V) glu + sulf	n.d.	n.d.	4722	7770	5195
12-Hydroxy-5,6-dehydrokavain (IX) free	40	50	70	71	29
12-Hydroxy-5,6-dehydrokavain (IX) glu	38	53	68	74	34
12-Hydroxy-5,6-dehydrokavain (IX) glu + sulf	35	50	64	65	30
6-Phenyl-hex-5-ene-2,4-dione (XV)	n.d.				
Unidentified metabolite (XVIII) free	n.d.	277	940	1217	355
Unidentified metabolite (XVIII) glu	350	530	1196	1494	549
Unidentified metabolite (XVIII) glu + sulf	353	572	1190	1495	540

Kavain and hydroxykavain (I) were not found in all urine samples. The metabolite 12-hydroxykavain (III, *RRT*: 0.43), 12-hydroxy-7,8-dihydrokavain (V, *RRT*: 0.41) and 12-hydroxy-5,6-dehydrokavain (IX, *RRT*: 0.55) were detected in all of the urine samples up to 24 h. The unidentified metabolite (XVIII *RRT*: 0.30) was detected in urine from the first 2 h up to 24 h. Its UV spectrum is related to 12-hydroxykavain, but shows an early retention time.

The metabolite 12-hydroxykavain (III) was found in the urine in its free, glucuronide and sulfate form. The maximum concentrations of its free and sulfate derivatives were reached after 4 to 8 h, and for its glucuronide form after 8 to 18 h. The metabolite 12-hydroxy-7,8-dihydrokavain (V) was not detected in its free form up to 24 h. The glucuronide and the sulfate derivatives of 12-hydroxy-7,8-dihydrokavain (V) first appeared after 4 to 8 h from the oral uptake with maximum concentrations after 8 to18 h. The metabolite 12-hydroxy-5,6-dehydrokavain (IX) was detected in relative small concentrations and only in its free form, no conjugations were observed. The metabolite 6-phenyl-5-hexene-2,4-dione (XV) was not detected in the urine samples by means of our HPLC-DAD method. The unidentified metabolite (XVIII) was found as free and conjugated derivative. Presumably the metabolite (XVIII) is excreted in its free and glucuronide form only (Table 13).

3.2.2.2 Determination of kavain and its metabolites in serum and blood using the HPLC-DAD method

The determination of kavain and its metabolites in serum and blood samples was carried out after collection of blood (10 ml divided into 2 glass tubes, 5 ml each, one of the glass tubes is centrifuged at 3000 rpm to collect the serum samples). The blood samples have been taken at the time course of 15, 30, 45, 60, 75, 90, 105, 120, 150, 180 and 240 minutes after a single oral dose of 800 mg of kavain. The analyses of the serum and blood samples were done before and after enzymatic cleavage (see page 35, chapter 2.7.6.2).

The serum concentration ranged between 10 and 40 ng/ml for kavain (K), 11and 50 ng/ml for hydroxykavain (I), 29 and 107 ng/ml for 12-hydroxykavain (III glucu+sulf) and 38 and 84 ng/ml for the unidentified metabolite (XVIII).

The metabolites 12-hydroxy-7,8-dihydrokavain (V) and 6-phenyl-5-hexene-2,4-dione (IX) were not detected in serum (Table 14).

Table 14: Serum concentrations of kavain and its metabolites in their free and conjugated form after a single oral dose (800 mg) of D,L-kavain (glu: glucuronide, sulf: sulfate)

	Concentration in ng/ml										
Time in minutes	**15**	**30**	**45**	**60**	**75**	**90**	**105**	**120**	**150**	**180**	**240**
d,l-kavain (K)	n. d.	15	40	21	23	19	17	18	15	12	10
Hydroxykavain (I)	n. d.	11	21	38	50	34	28	26	22	20	15
12-Hydroxykavain (III) free	n. d.	29	42	35	36	21	22	22	11	5	1.5
12-Hydroxykavain (III) glu	n. d.	60	96	83	85	63	53	58	37	36	31
12-Hydroxykavain (III) glu + sulf	n. d.	62	107	96	91	71	73	70	40	36	29
Unidentified metabolite (XVIII)	n. d.	40	52	63	68	76	84	80	59	47	38
12-Hydroxy-7,8-dihydrokavain (V) free	not detected										
12-Hydroxy-7,8-dihydrokavain (V) glu	not detected										
12-Hydroxy-7,8-dihydrokavain (V) glu + sulf	not detected										
6-Phenyl-5-hexen-2,4-dione (XV)	not detected										
12-Hydroxy-5,6-dehydrokavain (IX) free and conjugate (glu+sulf)	detected but not quantified										

The metabolite hydroxykavain (I) was detected in blood, serum but not in urine. Up to now no data about hydroxykavain (I) occurrence and concentration in blood have been published.

In our experiment hydroxykavain (I) was detected in serum at concentrations of 11-50 ng/ml. Its maximum was observed 2.5 h after the oral dose of kavain. Due to the absence of reference substance, we identified it according to the GC/MS

fragmentation and the DAD UV spectrum of the HPLC. Its UV spectrum is related to kavain and 12-hydroxykavain (Figure 39).

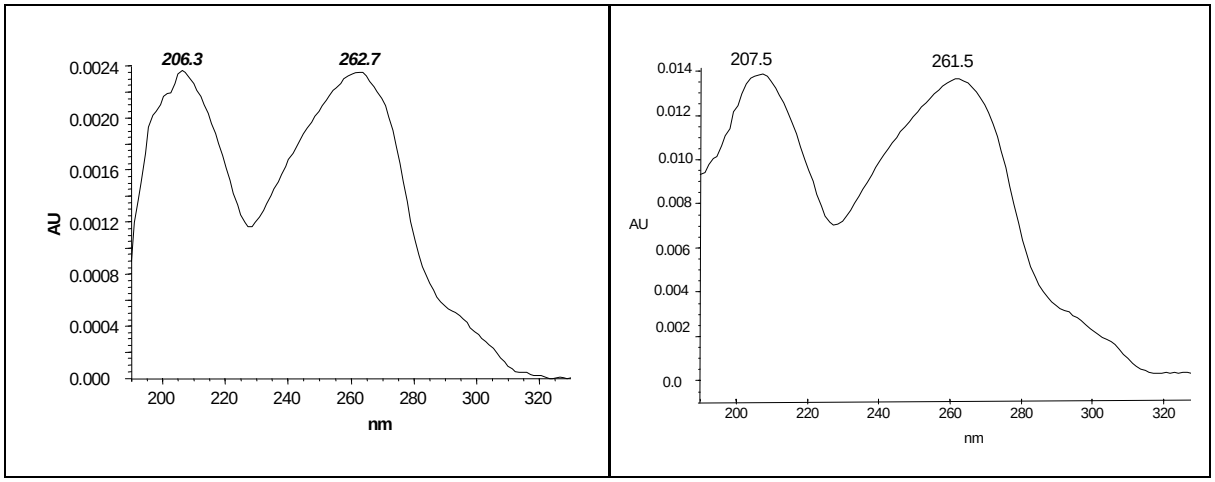


Figure 39: UV-spectrum of hydroxykavain (I) left and 12-hydroxykavain (III) right

The following diagram shows the effect of the enzymatic hydrolysis on 12-hydroxykavain (III) using ß-glucuronidase from E. coli and ß-glucuronidase / aryl sulfatase from H. pomatia.

We have selected 12-hydroxykavain because of its high concentrations in blood and serum. 12-Hydroxykavain was detected in serum in its free, glucuronide and sulfate form. The analyses of the blood or serum proved that 12-hydroxykavain (III) can not only be found in its free form but also as its sulfate and glucuronide derivatives. The 12-hydroxykavain derivatives appeared in serum and blood after 30 min, and reached their maximum 45 min after oral uptake.

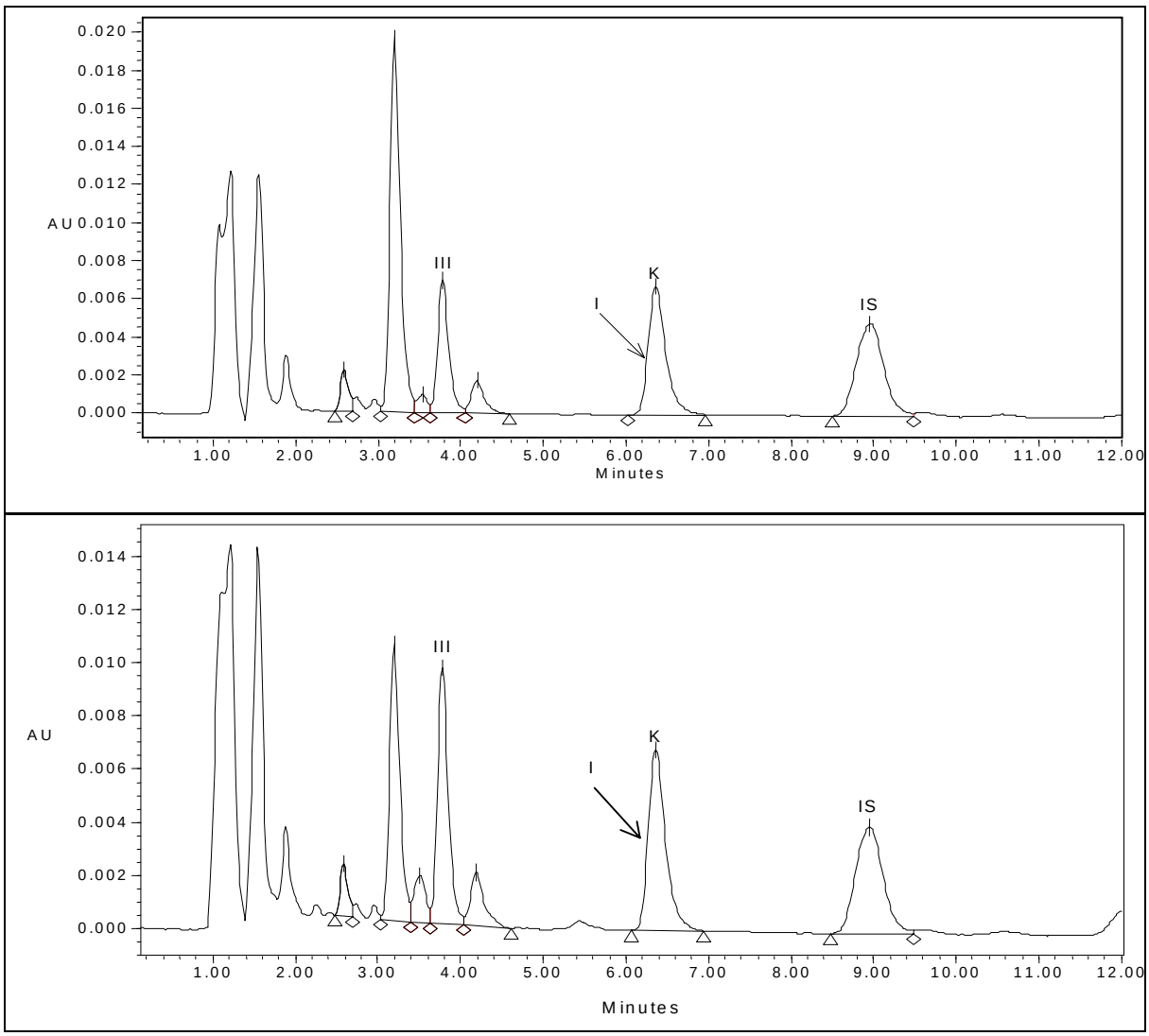


Figure 40: HPLC chromatogram of serum sample 60 min after the oral uptake of 800 mg kavain. Hydroxykavain (I), 12-Hydroxykavain (III) and kavain (K) (top without and bottom with incubation with E. coli)

The real concentrations of the 12-hydroxykavain conjugated derivatives were calculated throughout the total amount detected in serum. The glucuronide form presented the highest amount among the others, and showed that it is still detectable in blood 4 h after application. The sulfate form seems to have disappeared after 3 h (Figure 41).

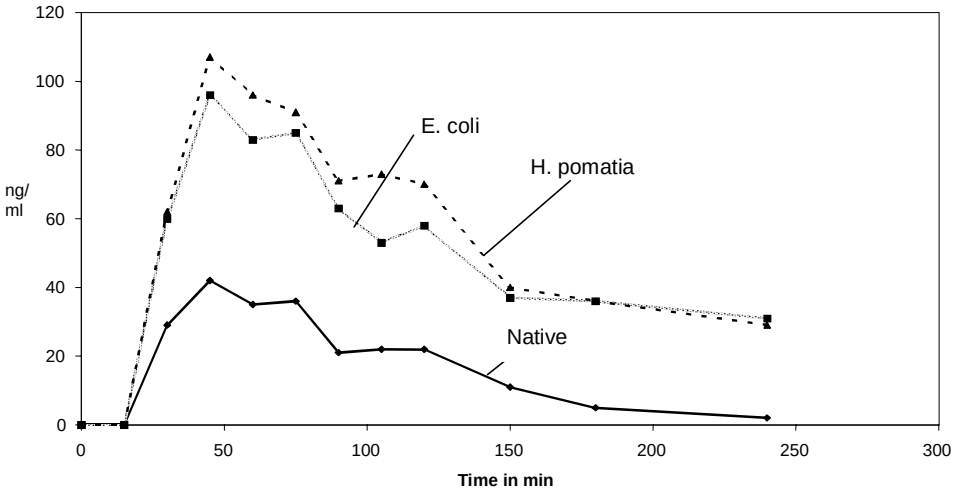


Figure 41: 12-Hydroxykavain in serum in its free and conjugated forms with and without incubation with E. coli and H. pomatia enzymes

The summary of this experiment is that the HPLC-DAD method was used for the determination of kavain and its metabolites in human samples (blood, serum and urine). Kavain (K) and hydroxykavain (I) were found in blood and serum in their free form. 12-Hydroxykavain (III) was detected as free derivative as well as in form of its glucuronide and sulfate derivatives. 12-Hydroxykavain (III) was found in blood or serum up to 4 h and in the urine samples up to 24 h in all of its 3 derivatives. The metabolite 12-hydroxy-7,8-dihydrokavain (V) was not detected in blood and serum but only in the urine sample after 8 h from the oral dose. This metabolite has a UV spectrum which is not related to kavain and 12-hydroxykavain but with the specific UV (λ_{max}: 193; 227 nm, Figure 42) which is confirmed with its reference substance λ_{max} of 194.6; 226.2 nm (Figure 23).

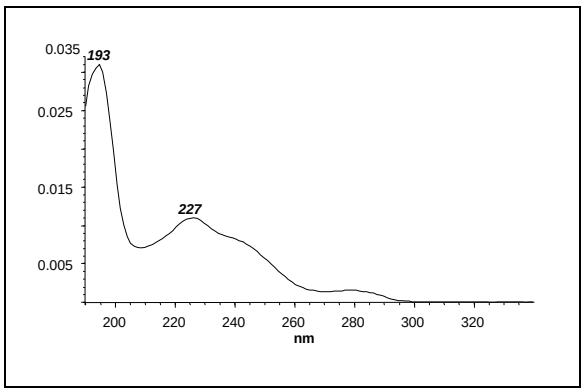


Figure 42: UV spectrum of 12-hydroxy-7,8-dihydrokavain

12-Hydroxy-7,8-dihydrokavain was not found in urine as free derivative but only the glucuronide and the sulfate derivatives. The metabolite 12-hydroxy-5,6-dehydrokavain was detected in blood, serum and urine in traces amounts as free derivative as well as its conjugated derivatives. The unidentified metabolite (XVIII) was found free only, its signal did not changed after the enzymatic hydrolyses. The main kavain human metabolites detected using the HPLC-DAD method are listed in table 15.

Table 15: Kavain and its metabolites in blood, serum and urine detected by HPLC-DAD (S: serum, B: blood, U: urine; * detected GC/MS and LC/MS

Substance	Free			Glucuronide			Sulfate		
	S	B	U	S	B	U	S	B	U
Kavain (K)	+	+	-	-	-	-	-	-	-
5,6-Dehydrokavain (VI)*	-	-	+	-	-	-	-	-	-
Hydroxykavain	+	+	-	-	-	-	-	-	-
12-Hydroxykavain (III)	+	+	+	+	+	+	+	+	+
12-Hydroxy-7,8-dihydrokavain (V)	-	-	-	-	-	+	-	-	+
12-Hydroxy-5,6-dehydrokavain (IX)	+	+	+	+	+	-	+	+	-
Unidentified metabolite (XVIII)	+	+	+	+	+	+	+	+	+

3.3 Application of the LC/MS method for the determination of kavain urinary metabolites

3.3.1 Detection of kavain and its metabolites in urine by LC/MS

LC/MS reference spectra for kavain (K) and its yet available metabolites 12-hydroxykavain (III), 12-hydroxy-7,8-dihydrokavain (V), 12-hydroxy-5,6-dehydrokavain (IX) and 6-phenyl-5-hexene-2,4-dione (XV) were needed for the LC/MS library. Therefore, in-source fragmentation of protonated molecules by electrospray-ionisation with collision induced dissociation (ESI/CID) was used by orifice voltage (OR) switching (20, 50, 80 V orifice-voltage) [Weinmann

et al. 1998]. For kavain the protonated molecule [MH^+] showed only low abundance m/z 231, whereas for the available metabolites base peak intensity was obtained for the protonated molecules with low orifice voltage. At higher voltages all compounds showed rich ESI/CID-mass spectra with characteristic fragment ions (Figure 43 - Figure 47).

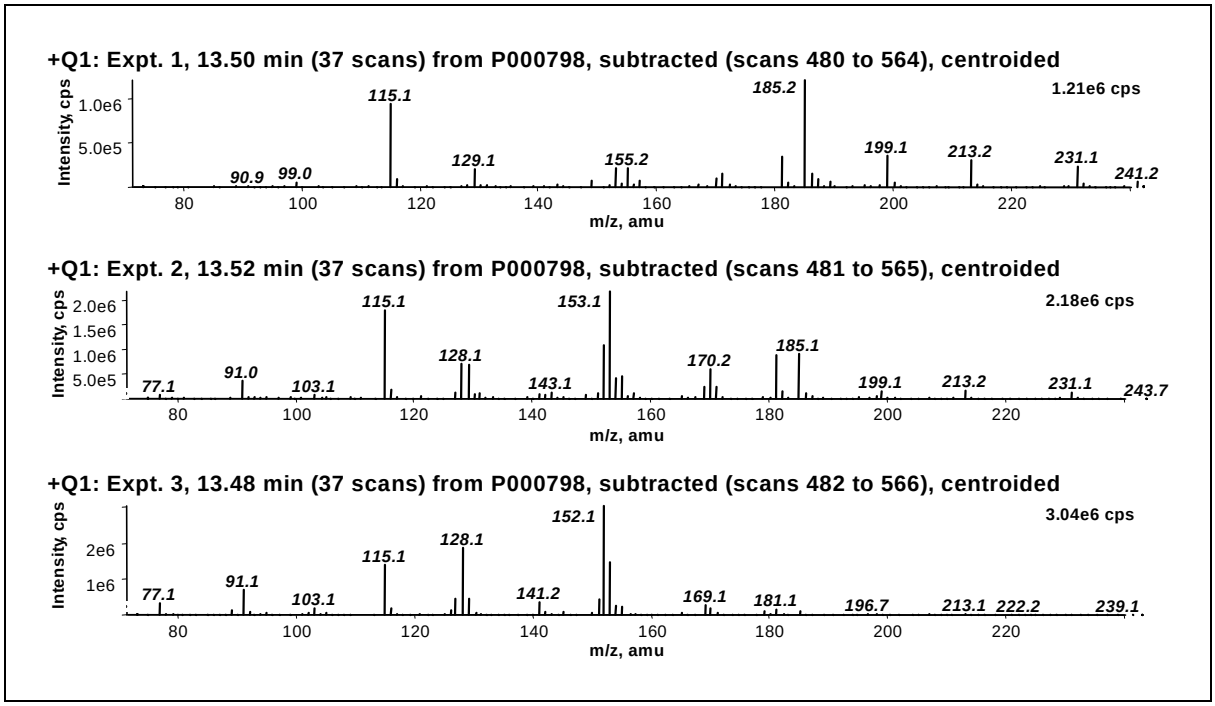


Figure 43: ESI/CID mass spectra of kavain (K) *RT*: 13.50 min: Orifice-voltages 20, 50 and 80 Volts, respectively (from top to bottom). Protonated molecule (m/z 231) and characteristic fragment ions (m/z 185, 153, 128, 115, 91, 77)

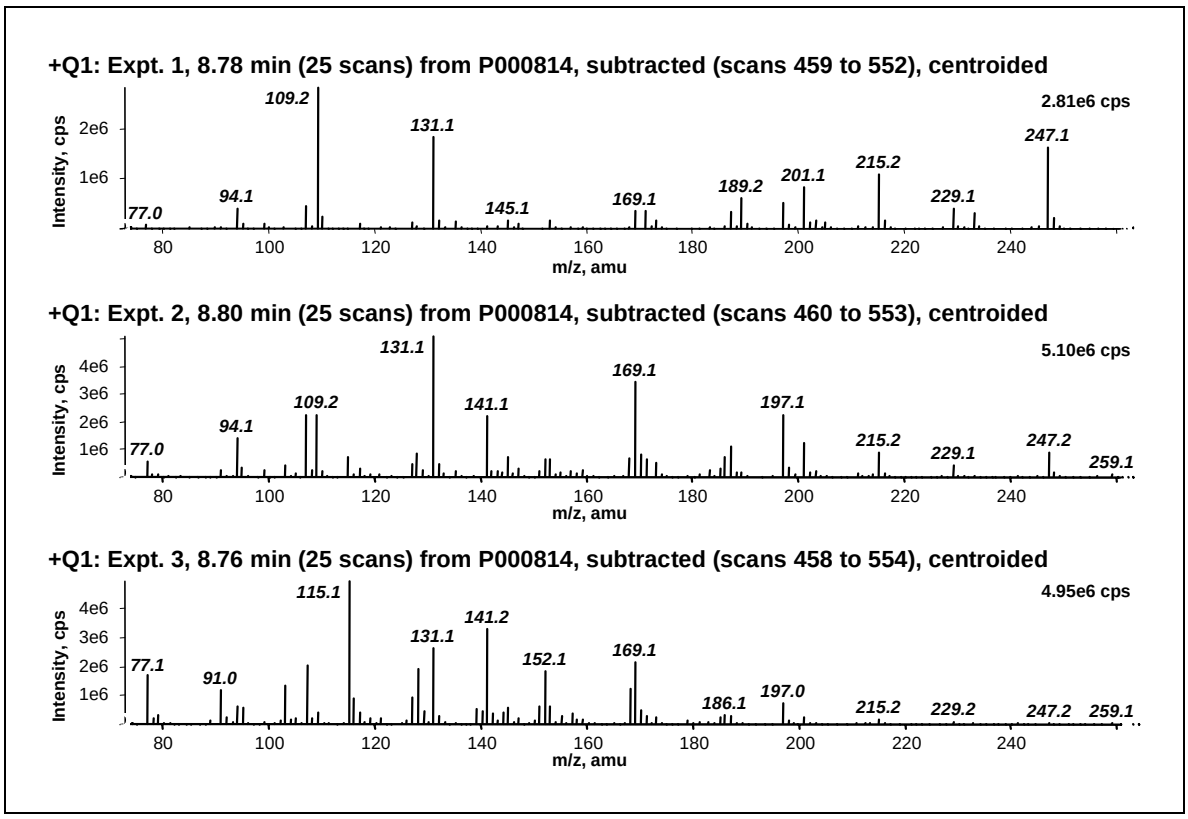


Figure 44: ESI/CID mass spectra of 12-hydroxy-kavain (III) *RT*: 8.78 min. Orifice-voltages 20, 50 and 80 Volts, respectively (from top to bottom). Protonated molecule (m/z 247) and characteristic fragment ions (m/z 215, 197, 169, 141, 131, 115, 109, 91, 77)

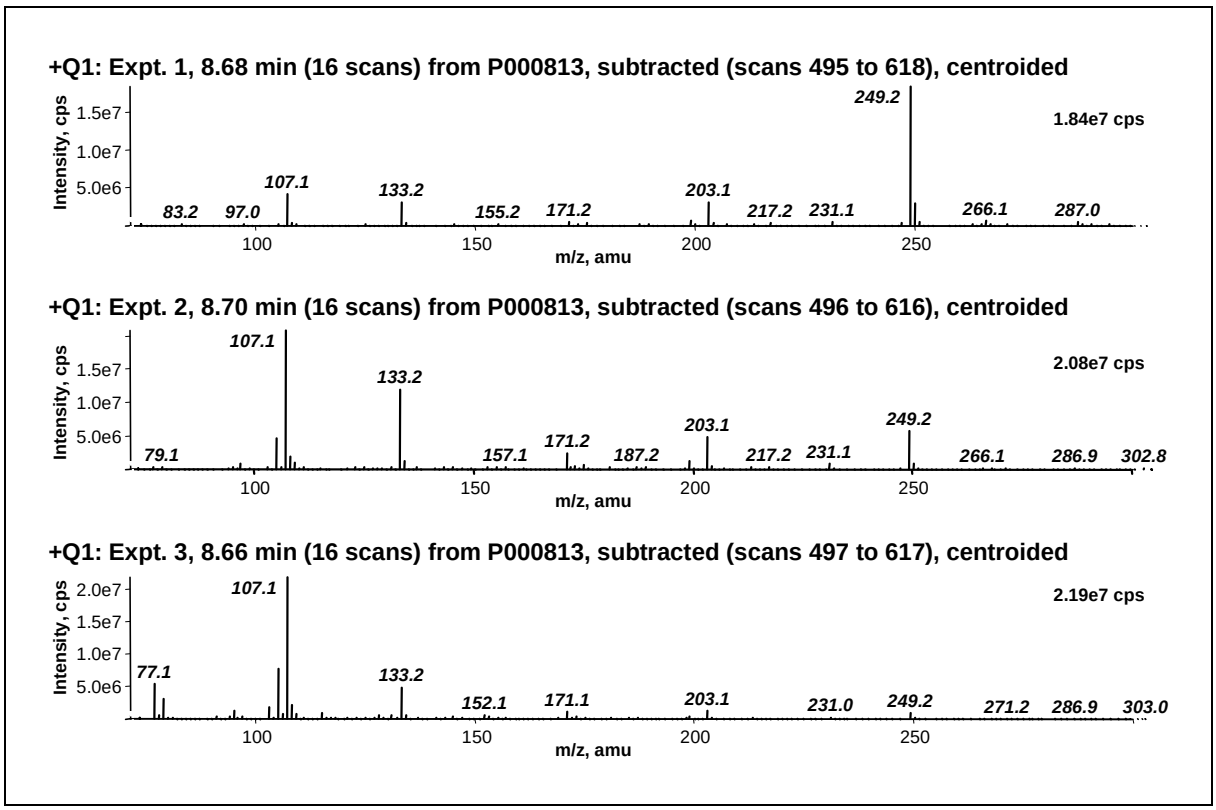


Figure 45: ESI/CID mass spectra of 12-hydroxy-7,8-dihydro-kavain (V) *RT*: 8.68 min Orifice-voltages 20, 50 and 80 Volts, respectively (from top to bottom). Protonated molecule (m/z 249) and characteristic fragment ions (m/z 203, 171, 133, 107, 77)

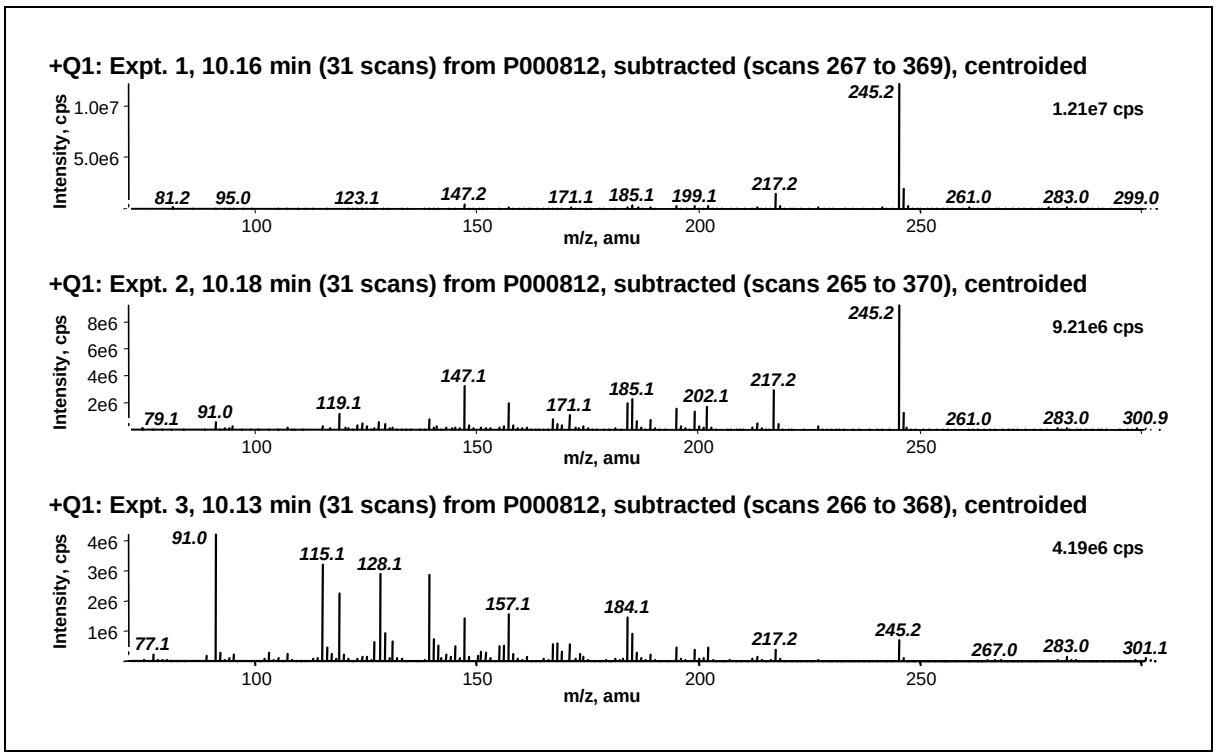


Figure 46: ESI/CID mass spectra of 12-hydroxy-5,6-dehydro-kavain (IX) *RT*: 10.16 min: Orifice-voltages 20, 50 and 80 Volts, respectively (from top to bottom). Protonated molecule (m/z 245) and characteristic fragment ions (m/z 217, 185, 147, 140-139, 128, 115, 91, 77)

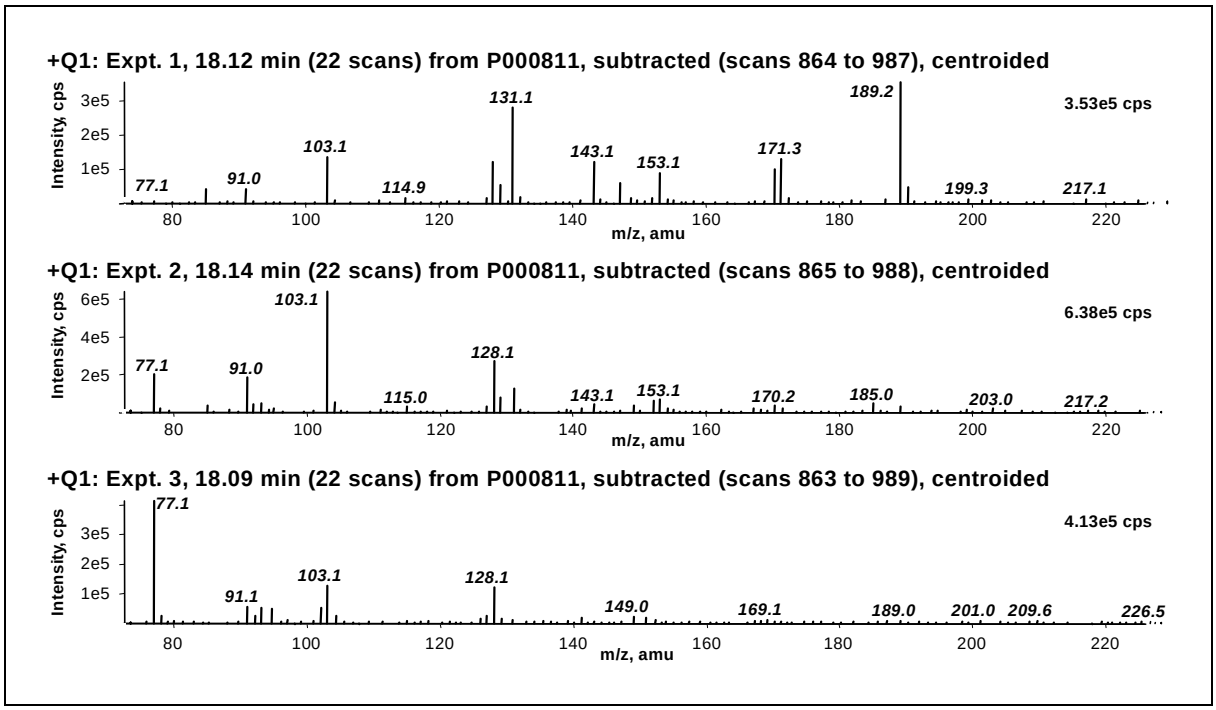


Figure 47: ESI/CID mass spectra of 6-phenyl-5-hexen-2,4-dion (XV) *RT*: 18.12 min: Orifice-voltages 20, 50 and 80 Volts, respectively (from top to bottom). Protonated molecule (m/z 189) and characteristic fragment ions (m/z 131, 128, 103, 91, 77)

3.3.2 Detection of kavain and its four main metabolites in urine samples after a single oral dose of 800 mg kavain by LC/MS

The LC/MS method was used for the confirmation of the HPLC-DAD results. The human urine samples (24 h urine) which was collected as described in page 31, chapter 2.7.1.3 was extracted and analysed by LC/MS after enzymatic hydrolyses using ß-glucuronidase from E. coli and ß-glucuronidase and arylsulfatase from H. pomatia. Kavain (K) and its metabolites 12-hydroxykavain (III), 12-hydroxy-7,8-dihydrokavain (V), 12-hydroxy-5,6-dehydrokavain and 6-phenyl-hex-5-ene-2,4-dione were detected by LC/MS with ESI/CID. The metabolite 5,6-dehydrokavain (VI) was detected in urine by LC/MS. 5,6-dehydrokavain was not available as reference substance, therefore in addition to the LC/MS analysis, 5,6-dehydrokavain was identified by product ion scan in triple-quadrupole mode (MS/MS) (Figure 48). The protonated molecule was isolated and fragmented by CID in the collision cell of the mass spectrometer.

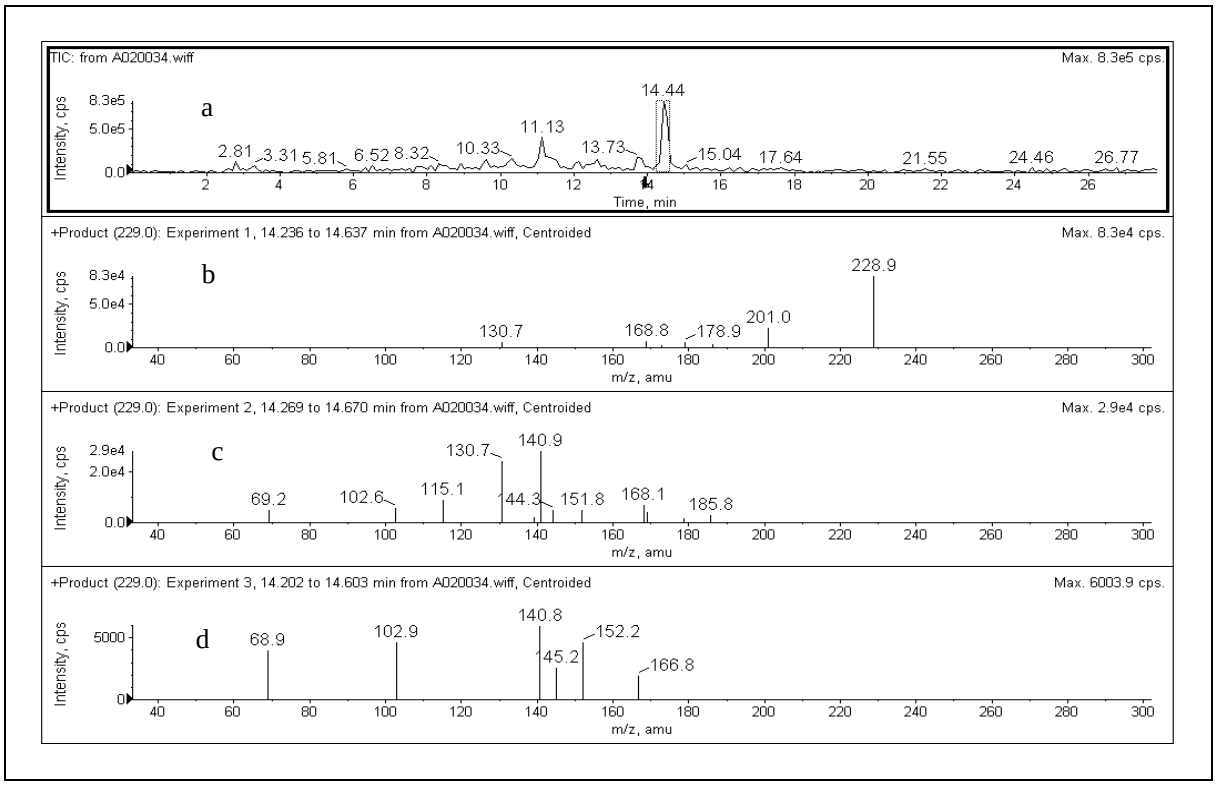


Figure 48: LC/MS-MS of 5,6-dehydrokavain (VI) *RT*: 14.44 min., a: TIC of product ion scan; b, c and d: product ion spectra of precursor [MH^+] m/z 229 with low, medium and high collision energy (CE)

A summary of LC/ESI-CID/MS and MS/MS spectral data of the urine metabolites of D,L-kavain is shown in table 16.

Table 16: LC/MS analyses of the reference compounds and the urine sample (collected in 24 h) after kavain oral uptake (* ESI-CID mass spectra of the detected metabolites in urine are shown as attachment. ** detected by using a solvent gradient programme)

Substance	**RT of urinary metabolites [min]**	**Prominent fragment of characteristic ions in decreasing order of ion-intensities. Molecular ion underlined***
p-Hydroxy-7,8-dihydrokavain (V)	10.83	203, 171, 133, 107, 231, 77, <u>249</u>
p-Hydroxykavain (III)	10.93	215, 197, 169, 141, 131, 115, 109, 91, 77, <u>247</u>
p-Hydroxy-5,6-dehydrokavain (IX)	11.43	217, 185, 147, 140, 139, 128, 115, 91, 77, <u>245</u>
Kavain (K)	13.84	185, 153, 128, 115, 91, 77,141, <u>231</u>
5,6-Dehydrokavain (VI)	14.44	103, 69, 115, 131, 141, 152, 186, 201, <u>229</u> (MS/MS)
6-Phenyl-5-hexen-2,4-dione (XV)	**	131, 128, 103, 91, 77, <u>189</u>

3.4 Kinetics of 12-hydroxykavain

The pharmaceutical preparation of kavain used in this study is marked under the trade name Neuronika ® (Klinge Pharma, Munich, Germany). Our study is based on kavain self medication. Four Neuronika ® capsules à 200 mg (= 800 mg) kavain were given in oral doses. Blood, serum and urine were collected at different times (page 31, chapter 2.7.1.3). The serum concentration ranged between 10 and 40 ng/ml for kavain (K), 11 and 50 ng/ml for hydroxykavain (I), 29 and 107 ng/ml for 12-hydroxykavain (III glucu+sulf) and 38 and 84 ng/ml for the unidentified metabolite (XVIII).

The metabolites 12-hydroxy-7,8-dihydrokavain (V) and 6-phenyl-5-hexene-2,4-dione (IX) were not detected in serum (page 63, Table 14). 12-Hydroxykavain (III) was selected for the kinetics study after oral kavain dose.

3.4.1 Kinetic of 12-hydroxykavain in serum

All three forms (free, sulfate and glucuronide) of 12-hydroxykavain (III) start to appear in serum after a rather short lag time of 30 min (0.5 h), peaking after 45 min (0.75 h), which indicates a high metabolic turnover. The total amounts of the glucuronide (B) and sulfate (C) metabolite concentrations are both 2 to 2.5 fold higher in serum than the one of the free form (A). Relatively high concentrations of the kavain-metabolites indicate, that the period of 4 h between kavain uptake, and the last sample collection seems to be too short to access the complete kinetic time course as there are relatively high concentrations left at this time

The half-lives of the terminal slope phase ranged from 0.7 to 1.9 h, indicating that at least the bound forms may be found in serum up to ~ 10 h after an oral dosage of kavain.

Pharmacokinetics studies on C14-labelled D,L-kavain were carried out on rat after an intravenous as well as oral doses. The study revealed that 78 % of the given D,L-kavain was good absorbed and 98 % of the absorbed amounts which are metabolised with the first pass effect [Klinge Pharma 1995].

The main metabolite of kavain in blood and serum is 12-hydroxykavain (III) which was detected at its maximum (45 min after oral intake) in serum as 50 % glucuronide, 12 % sulfate and 38 % free form (page 66, Figure 41), with a serum / blood ratio of 2:1.

Apparently the volume of the distribution cannot be interpreted for the kavain in blood. The values given assume 100 % bioavailability and 100 % turnover to the metabolite forms, which does not seem to be realistic. The same holds for the clearance data evaluated as these rely on the volume of distribution. Because of the sparse data set, one and two compartment models could not be discriminated. It is suspected that a two compartment model will be more appropriate if concentrations are measured over at least 10 hours.

The time courses of the free and conjugated forms of III were analysed by non-compartmental analysis using WinNonLin 3.4 (Pharsight, USA). The results are documented in table 17. The kinetics data are based on the kavain oral dose.

Table 17: 12-Hydroxykavain in *serum* (free and conjugated forms); A: free form; B glucuronide form; C glucuronide and sulfate form. (T lag: lag time before onset of kinetics; T max: time of maximal concentration; C max: maximal concentration; C last concentration at T last; λ_z: rate constant of terminal slope; T 50: half-life during terminal slope; AUC_inf: area under concentration time curve *(extrapolated to infinity)*; Vz_F: apparent volume of distribution; MRT: mean residence time)

Parameters	Unit	A	B	C
T lag	h	0.5	0.5	0.5
T max	h	0.75	0.75	0.75
C max	ng/ml	41.0	94.0	107.0
C last	ng/ml	2.0	31.0	28.0
λ_z	1/h	0.98	0.369	0.45
T 50	h	0.71	1.88	1.55
AUC_inf	ng/ml*h	64.80	274.15	271.44
Vz_F	$L*10^3$	12.64	7.91	6.61
MRT	h	1.57	3.15	2.72

3.4.2 Kinetic of 12-hydroxykavain in blood

Determination of 12-hydroxykavain in blood was based on the theory that if no serum samples are available, we could still be able to study the kinetic of this metabolite. The first observation between the serum and blood concentrations of 12-hydroxykavain was that the blood concentrations were about 50 % of the serum concentration, which correspond to various evaluations of serum to blood ratios of about 2:1. The second observation was that the concentration of the free 12-hydroxykavain (III-A) was at double concentration in the last sample (4 h after oral dose of kavain) than in serum. The third observation was that the half-life during terminal slope (T50) in blood was higher than in serum. The fourth observation was that the apparent volume of distribution (Vz_F) and the mean residence time (MRT) were higher in blood than in serum with a ratio of 2:1.

All three forms (free, sulfate and glucuronide) of 12-hydroxykavain (III) start to appear in blood after a rather short lag time of 0.25 h, peaking after 45 min at 0.75 h, which indicates a high metabolic turnover. The total amounts of the glucuronide (B) and

sulfate (C) metabolite concentrations are both 2 to 2.5 fold higher in blood than the one of the free form (A). Relatively high concentrations of the kavain-metabolites indicate that the period of 4 h between kavain uptake and the last sample collection seems to be too short to access the complete kinetic time course, as there are relatively high concentrations left at this time

The half-lives of the terminal slope phase ranged from 1.28 to 1.4 h, indicating that at least the bound forms may be found in blood up to ~ 10 h after an oral dosage of kavain.

Table 18 shows the pharmacokinetic of 12-hydroxykavain if blood instead of serum is analysed.

Table 18: 12-Hydroxykavain in *blood* (free and conjugated forms); A: free form; B glucuronide form; C glucuronide and sulfate form. (T lag: lag time before onset of kinetics; T max: time of maximal concentration; C max: maximal concentration; C last concentration at T last; λ_z: rate constant of terminal slope; T 50: half-life during terminal slope; AUC_inf: area under concentration time curve *(extrapolated to infinity)*; Vz_F: apparent volume of distribution; MRT: mean residence time

Parameters	**Unit**	**A**	**B**	**C**
T lag	h	0.5	0.5	0.5
T max	h	0.75	0.75	0.75
C max	ng/ml	20.0	53.0	62.0
C last	ng/ml	4.0	11.0	13.0
λ_z	1/h	0.49	0.51	0.50
T 50	h	1.40	1.28	1.37
AUC_inf	ng/ml* h	33.96	112.34	145.23
Vz_F	$L*10^3$	47.70	13.17	10.90
MRT	h	2.74	2.26	2.53

3.4.3 Renal excretion data of 12-hydroxykavain

12-Hydroxykavain (III) was found in urine as its free, glucuronide and sulfate form. The ratio of the conjugated forms (sulfate to glucuronide) was observed up to 24 h. The ratio of sulfate to glucuronide decreased slightly from 4 (first urine samples) to 3

in the last sample after 24 h. In the 24 h urine sample, 12-hydroxykavain (III) was found as 1.4 % in its free form, 15.3 % as glucuronide form and 83.3 % as sulfate form. While renal excretion of free 12-hydroxykavain is in the same order of magnitude as creatinin clearance (~ 7.2 l/h) according to the creatinin concentrations of each urine sample of this study. The glucuronide and sulfate-metabolites of 12-hydroxykavain exhibit very high clearance values.

Excretion via urine seems to go on beyond 24 h as indicated by the relatively high amounts found during the last sample period.

The whole urine samples from 2 to 24 h revealed very low concentration of free 12-hydroxykavain (III-A) compared to the conjugated forms. The maximum concentrations of both glucuronide and sulfate conjugates of 12-hydroxykavain (B and C) were observed between 8 to 18 h which followed by a drop in their concentration up to the 24 h urine sample.

The total amount of the free and conjugated forms of 12-hydroxykavain in urine, which were 30.7 mg (0.125 mmol) over 24 h, represent 3.6 % of the given oral dose of 800 mg (3.478 mmol) kavain (Table 19).

Table 19: Urinary excretion of 12-hydroxykavain after kavain oral up take; A: free form; B glucuronide form; C glucuronide and sulfate form (Cl renal: renal clearance)

Time [h]	Unit	A	B	C
0-2	µg	91	1404	5954
2-4	µg	88	1043	4360
4-8	µg	82.5	1750	7690
8-18	µg	53.9	2765	9952
18-24	µg	18	787	2377
0–24	µg	334	7749	30333
Cl renal	l/h	9.8	68.9	208.8

4 Discussion

4.1 Analytical methods

The specific objectives of this study were to compare the metabolic pathways of kavain in Hep-G2 cells and in humans with the previously described rat [Rasmussen et al. 1979, Scheline 1991] and human in vivo models [Duffield et al. 1989, Köppel and Tenczer 1991, Klinge Pharma 1995] and to identify and characterise major or yet unknown metabolites in human.

The biological samples and the reference compounds used for this study were analysed by means of GC/MS before and after derivatisation. The advantage of GC/MS is its great separation power and the availability of sophisticated spectra libraries. Therefore it was used for screening to find new metabolites of kavain. The disadvantages of this method is that only thermostable and gaseous substances can be measured. Therefore derivatisation is needed very often, which makes it difficult to detect unknown metabolites. In this work two derivatisation procedures (silylation and methylation) were needed to analyse the different metabolites. A further problem is the difficulty to quantify substances, if no deuterated reference substances are available. The usage of d_5-diazepam proved, throughout the precision tests, that it is not sufficient to be recommended as an internal standard for a quantitative analyses.

Therefore HPLC was the alternative analytical procedure used for the detection of thermolabil and high molecular metabolites and for quantitative analyses needed for the kinetics. HPLC-DAD made it possible to detect and determine kavain and its metabolites with high recovery, good precision and short time analyses. The DAD UV spectra helped in the orientation required during the research for new products related to kavain and its metabolites. The quantitation of kavain (K), 12-hydroxykavain (III), 12-hydroxy-7,8-dihydrokavain (V) and 12-hydroxy-5,6-dehydrokavain (IX) (reference substances were available) and hydroxykavain (I), 5,6-dehydrokavain (VI) and the unidentified metabolite (XVIII) (no reference substance were available) in blood, serum and urine was carried out using HPLC-DAD eluent system [Daldrup et al. 1982, Pragst et al. 2001]. The quantitation was successful by using the specific wavelengths for each substance as described

on page 24, chapter 2.6. For the quantification of hydroxykavain, a special technique by raising of the column incubation temperature to 42 °C was used. The HPLC column was not suitable to detect the metabolite 6-phenyl-5-hexene-2,4-dione (XV).

LC/MS analyses were selected for the confirmation and identification of kavain metabolites especially in the case of kavain metabolites occurring at low concentrations. LC/MS reference spectra for kavain (K), 12-hydroxykavain (III), 12-hydroxy-7,8-dihydrokavain (V), 12-hydroxy-5,6-dehydrokavain (IX) and 6-phenyl-5-hexene-2,4-dione (XV) have been established. The last mentioned metabolite could only be identified when an isocratic LC-system was used. Since no reference substance of 5,6-dehydrokavain was available, its protonated molecule was isolated and fragmented by CID in the collision cell of the mass spectrometer. This study described for the first time the LC/MS and LC/MS-MS data of kavain and its main metabolites.

4.2 Kavain metabolism by means of Hep-G2 cell lines

The Hep-G2 cell culture was chosen as an in vitro model to study the kavain metabolism in human liver. The specific objectives of this study were to identify and characterise the kavain metabolite and to compare the metabolic pathways of this pharmacological active substance in Hep-G2 cells and in human. Several metabolic pathways were observed.

There was an extensive metabolism of D,L-kavain with these Hep-G2 cell lines. Fourteen kavain metabolites were detected and identified by GC/MS before and after derivatisation (silylation and methylation, page 47 and Table 5, Table 6 and Table 7). We found that the maximum production of metabolites occurred after an incubation time of 30 h to 70 h.

The Hep-G2 cell lines established from human hepatocellular carcinoma conserve differentiated characteristics, including certain pathways of drug biotransformation [Sata and Kato 1982, Patel et al. 1991, Neuman et al. 1993, Hayes et al. 1995, Poon et al. 1995]. In the present study it could be shown that in case of D,L-kavain, C_{12}-hydroxylation of the phenyl ring with the production of 12-hydroxykavain (III) predominated among the other biotransformation pathways. Beside hydroxykavain (I), 12-hydroxykavain (III), 11-hydroxy-7,8-dihydro-kavain (IV),

11-hydroxy-7,8-dihydro-kavain glucuronide (IVa), 12-hydroxy-7,8-dihydroavain (V) an unidentified metabolite (XVIII) were detected in the cell extracts. For the identification of these metabolites, mass spectra from the literature were used [Rasmussen et al. 1979, Duffield et al. 1989, Köppel and Tenczer 1991, Klinge Pharma 1995]. In addition to the hydroxylated metabolites a second group can be defined as end products of the lactone ring opening. In the Hep-G2 cell extracts 6-phenyl-3-hexene-2-one (XIV), 6-phenyl-5-hexene-2,4-dione (XV) and 4-hydroxy-6-hydroxyphenyl-5-hexene-2-one (XIX) were detected.

Grant et al. [1988] and Dawson et al. [1989] have reported that the Hep G2 cell lines have UDP-glucuronyl transferase activity. Our study proved the formation of glucuronide conjugate of the hydroxylated kavain metabolites. Thus the peak signal of 11-hydroxy-dihydro-kavain (IVa) was significantly increased after the enzymatic hydrolysis with ß-glucuronidase from E. coli corresponding to other hydroxylated kavain metabolites. Therefore there is no doubt that the Hep-G2 cells were able to produce glucuronide conjugates of some hydroxylated kavain metabolites.

It was interesting that the Hep-G2 cells were able to produce dehydro-derivatives of kavain metabolites (5,6-dehydrokavain VI) after incubation with the cell culture. This proves that the Hep-G2 cells are able to metabolise kavain the same way as humans through dehydratase enzymes, although the absence of the microsomal activities in the Hep-G2 cells [Schwartz 1981, Schwartz and Rup 1983, Huber et al. 1986]. The metabolite 4-oxy-cinnamyl acetone (XIII) was detected only in the cell culture but not in urine. The metabolite 6-phenyl-5-hexen-2,4-dione (XV) which has the identical chemical structure like 4-oxy-cinammyl acetone (XIII) was detected only in urine but not in blood. We could find no explanation for the phenomena. Both of these metabolites were detected only by the GC/MS method.

During this in vitro study on D,L-kavain metabolism we did not observe any damage or destruction of the Hep-G2 cells when they have been incubated up to 70 h with D,L-kavain. To give an answer to the question if kavain or one of the kava lactones have liver toxic effects we recommend that the kava lactones should be single incubated with our in vitro model to observe if any of these lactones cause damage of the Hep-G2 cells. Generally the aim of our study was to compare the in vitro and in vivo metabolic pathways of D,L-kavain. The findings of the in vitro models are consistent with the results of our current metabolism studies using the Hep-G2-cells

model. This is indicating that in vitro studies could be extrapolated to the in vivo metabolism.

4.3 Kavain metabolism in human

The self medication experiments were done by administration of oral doses of 200 and 800 mg D,L-kavain respectively.

We started our experimental trials to study the kavain metabolism by giving kavain in a single oral dose of 200 mg. The purpose was to guide the strategy of this study and to compare it with the metabolism data of the product information from Klinge Pharma. Blood and urine were collected in different time courses to study the human metabolism of kavain after oral uptake. Kavain (K) and hydroxykavain (I) were detected in serum and blood 45 min after the oral uptake. Hydroxykavain was detected up to 6 h and then disappeared in the last two samples (12 and 24 h). 12-Hydroxykavain (III) was detected first after 1,5 h after oral dose in its free form. It was detectable up to 12 h. 12-Hydroxy-7,8-dihydrokavain (V) was not found in blood and serum. In the urine samples 12-hydroxykavain was detected but no kavain (K). No kinetics data were obtained from this trial.The previous studies on kavain metabolism in man involved particularly the urinary metabolites [Duffield et al. 1989, Köppel and Tenczer 1991, Klinge Pharma 1995]. No data were published about the blood or serum kavain and its metabolites except those obtained from the products information of Klinge Pharma company [Klinge Pharma 1995] or the work from Johnson et al. 1991 who found that the maximum kavain plasma concentration was 70 ng/ml 3 h after oral uptake of 200 mg kava extract. This result is not comprehensible. Due to the absence of the description of the analytical method used for the determination of the kava lactones in plasma it is not possible to check if the results are valuable. Because kavain and hydroxykavain (I) are difficult to separate, it could be possible that hydroxykavain was detected and quantified instead of kavain. Other studies were done only on the identification of the urinary metabolites in animal (rats) after administration of oral kava lactones beverage [Rasmussen et al. 1979].

Up to now no data about hydroxykavain (I) occurrence in blood have been published. In our experiment hydroxykavain (I) was detected in serum at concentrations of 11-50 ng/ml. Its maximum was observed 2.5 h after the oral dose of kavain. Due to

the lack of hydroxykavain reference substance, we identified it according to the GC/MS fragmentation and the DAD-UV spectrum of the HPLC. Its UV spectrum is related to 12-hydroxykavain. Additionally its occurrence in urine was confirmed by LC/MS and LC/MS-MS.

Our final self medication study on kavain metabolism was carried out after application of 800 mg D,L-kavain as single oral dose. Blood samples were collected after 15, 30, 45, 60, 75, 90, 105, 120, 150, 180 and 240 minutes. Urine samples were collected before kavain uptake for the control studies, followed by the collection of urine after 2, 4, 8, 18 and 24 h. From the whole urine samples a 24 h urine sample was collected. All samples were stored at - 20 °C till analyses. Kavain itself could not be detected in urine. It was detected only in blood from 30 min up to 4 h (last sample) at quite low concentrations (10 to 40 ng/ml). It seems that kavain is quickly metabolised through the first pass effect mechanism [Hänsel and Kammerer 1996]. Klinge Pharma [1995] reported in its product information an animal investigation (rats) with ^{14}C labelled D,L-kavain after oral and i. v. route of administration. According to this information 98 % of this absorbed amount are metabolised with the first pass effect.

The main metabolite of kavain in blood and serum was found to be 12-hydroxykavain (III) (Figure 49), which was detected at its maximum 45 min after oral uptake. The maximum of III was observed at 45 min to 1 hour when a high single dose of D,L-kavain (800 mg) was applied. In serum 50 % of this metabolite was found as glucuronide, 12 % as sulfate and 38 % in its free form. The concentrations ranged between 29 and 107 ng/ml (page 63, Table 14); serum / blood ratio was found to be 2:1.

Klinge Pharma reported in the products information that in case of the administration of a single oral dose of 200 mg kavain to human, 12-hydroxykavain in form of its sulfate reached its maximum 104 min after uptake, compared to 45 min in our results, and showed a mean elimination half-life of 29 h [Klinge Pharma 1995]. The lack of detailed data from Klinge Pharma product information makes it difficult to accept or to reject these kinetic data. Our study was a pilot study on D,L-kavain metabolism and kinetics, therefore the observed data depended on the biotransformation profile of that volunteer person. The three individual trials using the high single dose of kavain (800 mg) showed that the maximum of the metabolite 12-hydroxykavain (III) ranged

between 45 and 60 min.

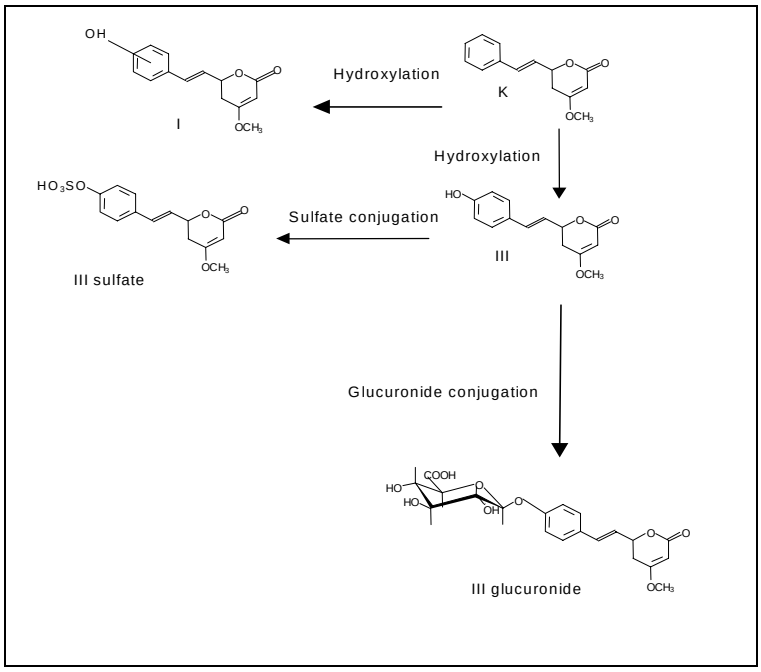


Figure 49: Hydroxylation of D,L-kavain (K) to hydroxykavain (I) and 12-hydroxykavain (III), its main metabolite in blood and formation of its conjugates

12-Hydroxykavain (III) is excreted in the urine in its free, glucuronide and sulfate form. The ratio of sulfate to glucuronide decreased slightly from 4 to 1 (first urine samples, 2 h after oral uptake) to 3:1 in the last sample after 24 h from the oral dose. In the 24 h urine sample, 1.4 % of 12-hydroxykavain (III) was found in its free form, 15.3 % as glucuronide and 83.3 % as sulfate. These results are in accord to with what is reported in the product information of Klinge Pharma [1995]. Here they reported that after administration of a single oral dose of 200 mg kavain 12-hydroxykavain was found in urine as follows: the free form was 1.0 % and the conjugated forms were ~ 85 % sulfate and 15 % glucuronide.

When kavain is given orally, it additionally was metabolised to 5,6-dehydrokavain (VI). This metabolite first was detected by Köppel and Tenczer [1991] in human urine after oral application. Klinge Pharma reported in its product information that after a dose of 200 mg, 0.3 % of kavain was found as 5,6-dehydrokavain in urine. Duffield et al. [1989] detected the 5,6-dehydrokavain (=desmethoxyyangonin) in human urine after the oral administration of kava beverage and he defined it as a product of yangonin desmethoxylation. 5,6-dehydrokavain is a major kava lactone present in the root extract.

5,6-Dehydrokavain might be further metabolised by desmethylation of the 4-methoxy group of the lactone ring to yield the unidentified metabolite (XVIII), and/or by hydroxylation of the phenyl ring to yield 12-hydroxy-5,6-dehydrokavain (IX). The hydroxylation of the phenyl ring could also take place at another C-atom of the phenyl ring, most probably C-11 to yield 11-hydroxy-5,6-dehydrokavain (IXa) (Figure 50).

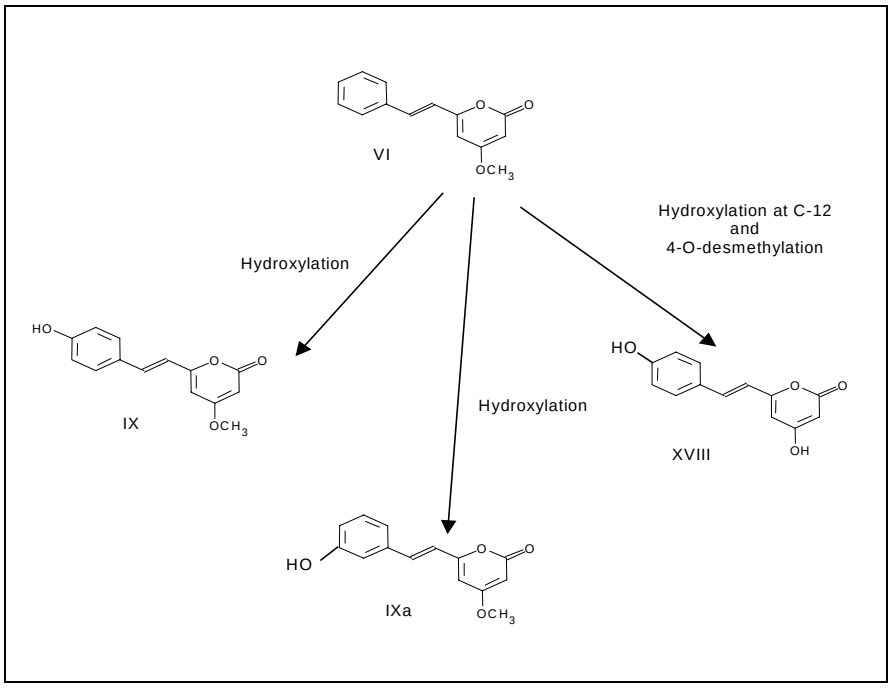


Figure 50: Proposed metabolic pathways profile of kavain to the 5,6-dehydro derivatives and the hydroxylation of kavain followed by 4-O-desmethylation

5,6-Dehydrokavain and these metabolites are belong to the dienolide kava lactone such as yangonin, therefore their UV spectra are not related to kavain or 12-hydroxykavain. The UV spectrum of 11-hydroxy-5,6-dehydrokavain is more similar to yangonin (Figure 51) due to the position similarity of the hydroxy and the methoxy function groups at the phenyl ring.

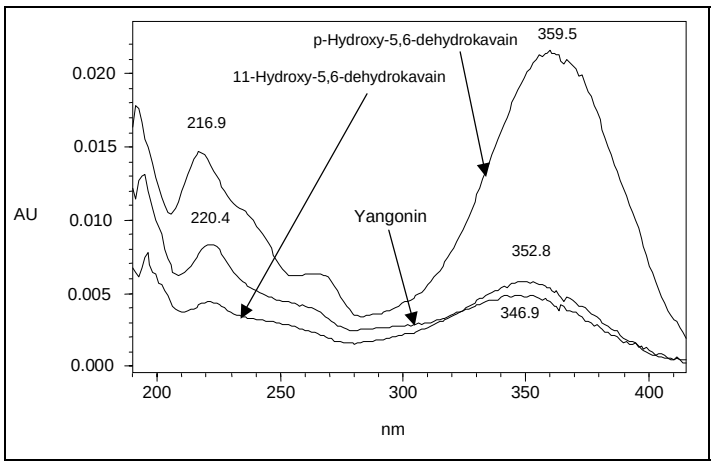


Figure 51: UV spectra of yangonin, 11- and 12-hydroxy-5,6-dehydrokavain

The unidentified metabolite (XVIII) has a UV spectrum which is related to 12-hydroxykavain and hydroxykavain with a maximum at 206.3 and 261.5 nm (Figure 52). This metabolite was detected in serum at concentrations between 38 to 84 ng/ml. The peak concentration was seen 2 h after kavain uptake (page 63 and Table 14). It was also detected in urine up to 24 h. The enzymatic hydrolyses revealed no changes on this compound, so we have no indication of conjugation of its hydroxy group.

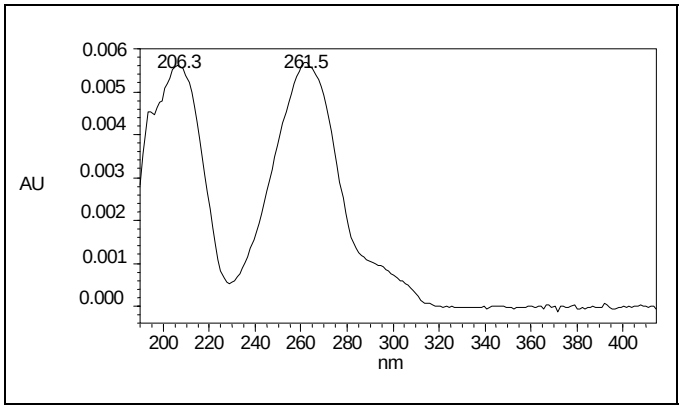


Figure 52: UV-spectrum of the unidentified metabolite (XVIII) detected in human serum/blood and urine

The hydroxylated metabolites 12-hydroxy-5,6-dehydrokavain (IX) and 11-hydroxy-5,6-dehydrokavain (IXa) were detected in serum, blood and urine at very low concentrations (below the limit of quantification), even after enzymatic hydrolyses (page 63 and Table 14). A differentiation between free and conjugated form was not possible. In urine IX and IXa were found at very low concentrations too (Table 13).

Another pathway of kavain metabolism is the formation of 7,8-dihydro derivatives. 12-Hydroxy-7,8-dihydrokavain (V) (available as reference substance) has a UV spectrum which is not related to kavain (K) or 12-hydroxykavain (III). The 11-hydroxy-7,8-dihydrokavain (IV) was described as rat urinary metabolite after oral administration of dihydrokavain [Rasmussen et al. 1979, Scheline 1991]. Data on its occurrence in blood were not available.

We were unable to detect 12-hydroxy-7,8-dihydrokavain (V) in blood or serum but we found it in urine. There it appeared not before 5 hours from kavain oral uptake, and only in its conjugated form with a glucuronide to sulfate ratio of 2 to 1 (page 61, Table 13). The absence of this metabolite in blood up to 4 hours after oral uptake could be explained due to the delayed reduction of the 7,8-double bond after the hydroxylation of the phenyl ring. We think that as soon as the hydroxylation and the reduction process took place, it will be immediately excreted in the urine. Another explanation for this observation is that the metabolite 12-hydroxykavain (III) is conjugated first by glucuronide and sulfate then followed with reduction of the 7,8-double bond to give the conjugated 12-hydroxy-7,8-dihydrokavain (glucuronide and sulfate form) which is excreted immediately (Figure 53).

Another reason might be that 12-hydroxy-7,8-dihydrokavain (V) is distributed into the deep compartments, and that its concentrations in blood are below the limit of detection. In this case it should be detectable in the corresponding tissues. No tissues of persons who died shortly after kavain uptake were available up to now to confirm or reject this thesis.

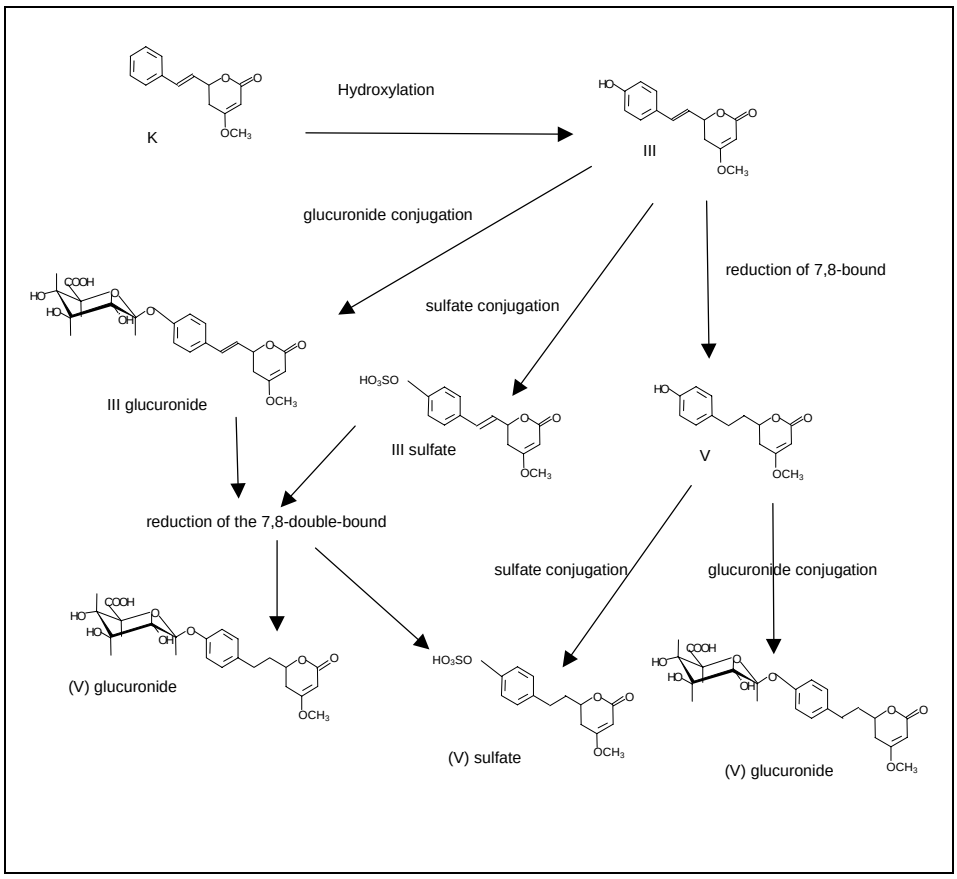


Figure 53: Proposed metabolic pathway of kavain (K) to form the 7,8-dihydro drivatives

Opening of the lactone ring and oxidation at side chain of degradation products at the C-9 of the phenyl ring as another metabolic pathway of kavain were also recognised. Furthermore the following metabolites were found when screening the extracts using the GC/MS method with and without derivatisation.

Kava acid methyl ester (X), cinnamyl acetone (XI), 4-hydroxycinnamyl acetone (XII), 6-phenyl-3-hexene-2-one (XIV), 6-phenyl-5-hexene-2,4-dione (XV), 4-hydroxy-6-hydroxyphenyl-5-hexene-2-one (XIX) and p-hydroxybenzoic acid (XVI). These metabolites were identified in the in vitro metabolism study of D,L-kavain using the Hep-G2 cells (page 48, Table 5, Table 6 and Table 7) and as D,L-kavain urinary metabolites in human (Table 9, Table 11 and Table 10).These metabolites were already described by Rasmussen et al. [1979], Duffield et al. [1989], Köppel and Tenczer [1991], Scheline [1991] and by Klinge Pharma [1995].

The proposed metabolic pathway of kavain to form 6-phenyl-5-hexene-2,4-dione (XV) by opening the lactone ring is presented in figure 54. This metabolite was found

in urine after silylation using the GC/MS method and by LC/MS. Its identity was confirmed by MS-MS.

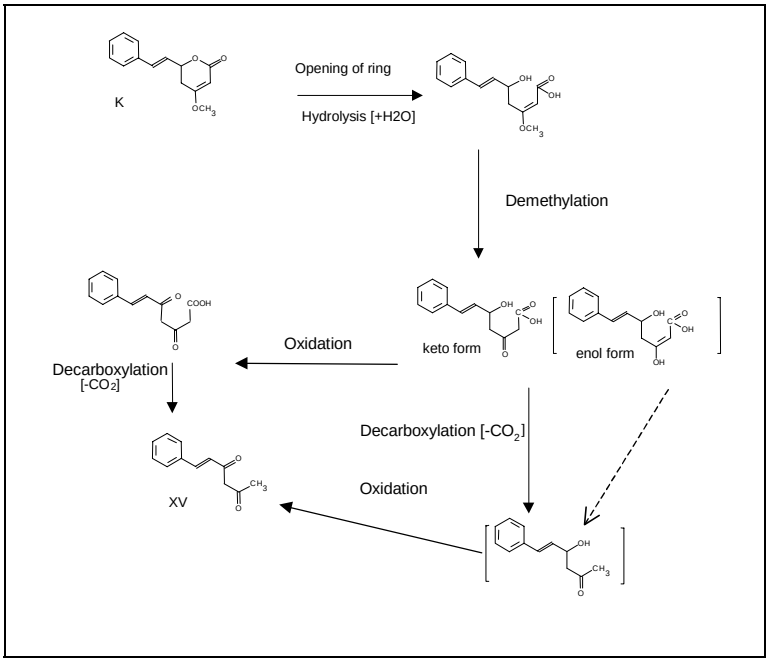


Figure 54: Proposed metabolic path way of kavain by opening of the lactone ring to give the metabolite 6-phenyl-hex-5-ene-2,4-dione

Because of the presence of kavain metabolites in blood at a relatively higher concentration than kavain itself, it is recommended not only to look for kavain but also for hydroxykavain (I), 12-hydroxykavain (III) and the metabolite (XVIII). This is valid especially in cases with an asserted intake of kavain where kavain itself could not be detected in blood, and when urine was not available. For example, we found in blood (after enzymatic cleavage) 4 to 10 times higher concentrations of these metabolites in comparison to kavain itself (page 63 and Table 14).

Due to the first pass effect, kavain is nearly completely metabolised. We think therefore, that not only kavain but at least one of its metabolites e.g. hydroxykavain (I), 12-hydroxykavain (III) and or the unidentified metabolite (XVIII) are pharmacologically active. In literature we didn't find any study about the effects of kavain metabolites but if we look to the similarity in the chemical structure of the metabolites found in blood and some of the major pharmacological active kava lactones as methysticin, yangonin and desmethoxyyangonin [Kretzschmar 1995, Hänsel and Kammerer 1996, Kaul and Joshi 2001, Czygan and Hiller 2002], we think that this assertion could be correct.

The tables 20 to 22 give an overview of kavain metabolites found in our study and those published in the previous literature.

The hydroxylated metabolites of kavain published in the previous literature [Rasmussen et al. 1979, Duffield et al. 1989, Köppel and Tenczer 1991, Scheline 1991 and Klinge Pharma 1995] were all detected by us in blood, urine and/or Hep-G2 cells with exception of 5-hydroxykavain (II). In serum and blood hydroxykavain (I) and 12-hydroxykavain in its free and conjugated form were the only hydroxylated metabolite that could be detected (Table 20).

Table 20: Hydroxylated and hydroxylated 7,8-dihydro derivatives of kavain metabolites

Symbol	Name	Hep-G2 cell	Blood / Serum	Urine
I	Hydroxy-kavain	+	+	-
II	5-Hydroxy-kavain	-	-	-
III	12-Hydroxykavain	+	+	+
III	12-Hydroxykavain-glucuronide	+	++	++++
III	12-Hydroxykavain-sulfate	+	++	++++
IV	11-Hydroxy-7,8-dihydro-kavain	+	-	+
IV	11-Hydroxy-7,8-dihydro-kavain-glucuronide	++	-	-
V	12-Hydroxy-7,8-dihydro-kavain	+	-	+
V	12-Hydroxy-7,8-dihydro-kavain-glucuronide	+	-	+++++
V	12Hydroxy-7,8-dihydro-kavain-sulfate	+	-	+++++

During this biotransformation study of kavain in human and in Hep-G2 cell lines we have observed that 5,6-dehydrokavain was detected but not its derivatives 12-hydroxy-5,6-dehydrokavain (IX) and 11-hydroxy-5,6-dehydrokavain (IXa).

The metabolites hydroxy-5,6-dehydrokavain (VII) and 7,8-dihydro-5,6-dehydrokavain (VIII) that were detected in the urine of rats [Rasmussen et al. 1979] were not found,

neither in blood / serum or urine nor in Hep-G2 cells. The unidentified metabolite XVIII could be a result of hydroxylation of 5,6-dehydrokavain with desmethylation at C-4 of the lactone ring. It was found in blood even 4 h after kavain uptake, and in urine up to 24 h (Table 21).

Table 21: 5,6-dehydro derivatives of kavain metabolites

Symbol	Name	Hep-G2 cell	Blood / Serum	Urine
VI	5,6-Dehydro-kavain	++	n.d.	++
VII	Hydroxy-5,6-dehydro-kavain	-	-	-
VIII	7,8-Dihydro-5,6-dehydro-kavain	-	-	-
IX	12-Hydroxy-5,6-dehydro-kavain	-	+	+
IXa	11-Hydroxy--5,6-dehydro-kavain	-	+	+
XVIII	Unidentified metabolite	++	++	++

The degradation products of kavain after opening of the lactone ring were detected in urine and the Hep-G2 cells only but not in blood and serum (Figure 55). Kava acid methyl ester (X) was found in human urine and Hep-G2 cells but not in blood. Köppel and Tenczer [1991] have detected the metabolite cinnamyl-acetone (XI) which is a decarboxylation product of kava acid methyl ester (X). It was not possible to detect it in our study at all. Only in urine we could detect the metabolite 4-hydroxy-cinnamyl-acetone (XII). The metabolite 4-oxy-cinnamyl-acetone (XIII) was found only in the Hep-G2 cells (Table 22).

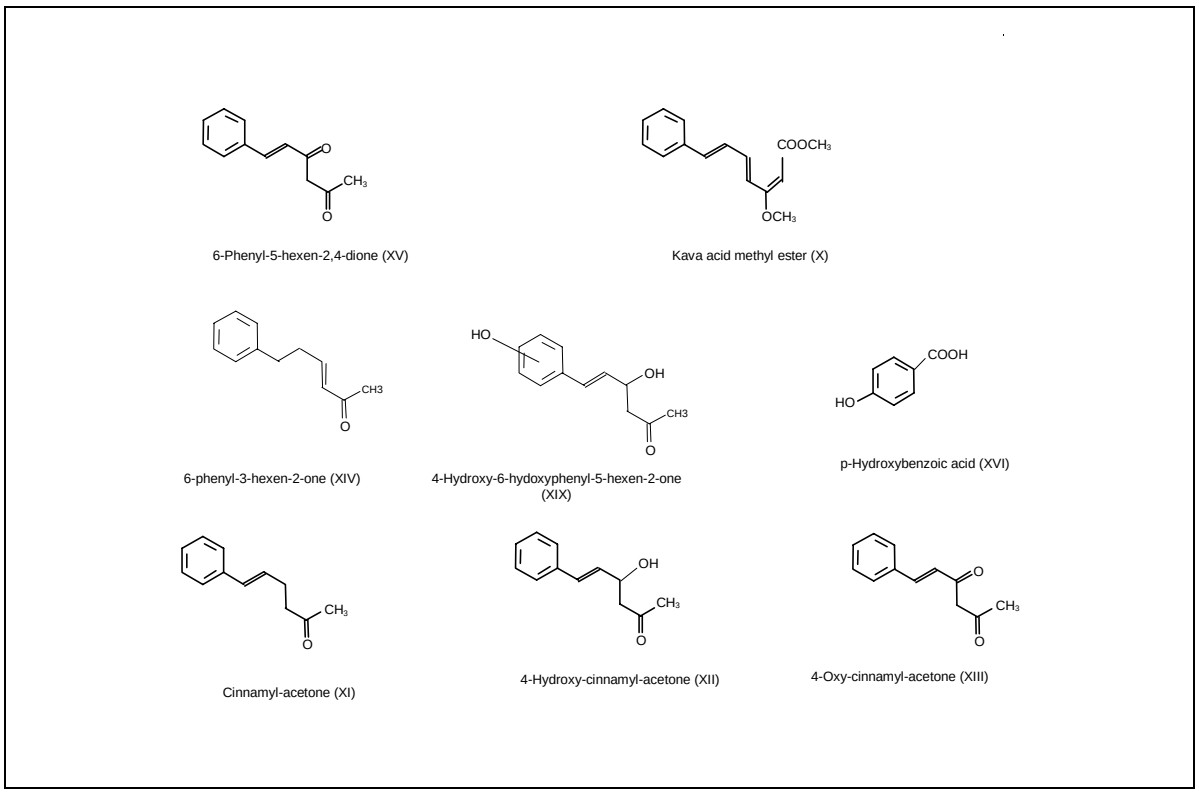


Figure 55: Degradation products of kavain after opening of the lactone ring

Table 22: Degradation products after opening of the lactone ring of kavain

Symbol	Name	Hep-G2 cell	Urine
X	Kava acid methyl ester	+	+
XI	Cinnamyl-acetone	+	+
XII	4-Hydroxy-cinnamyl-acetone	-	+
XIII	4-Oxy-cinnamyl-acetone	+	-
XIV	6-Phenyl-3-hexene-one	+	+
XV	6-Phenyl-5-hexene-2,4-dione	+	+
XVI	p-Hydroxybenzoic acid	+	+
XVII	Unidentified urinary metabolite [Rasmussen et al. 1979]	+	-
XIX	4-Hydroxy-6-hydroxyphenyl-5-hexen-2-one	+	-

5 Conclusion

Generally the aim of our study was to compare the in vitro and in vivo metabolic pathways of D,L-kavain. The findings of the previous reports are consistent with the results of our current metabolism studies using both the Hep-G2-cells and the human model. This is indicating that in vitro studies could be extrapolated to the in vivo metabolism.

Kavain metabolites have to be determined in blood. We presume that some of these metabolites should be pharmacologically active. Until now there is no evidence which proves their pharmacological effect.

In Germany the marketing of kavain and kava products is prohibited due to some liver diseases associated with kava consume. This might lead to less interest in this kavain metabolism study. We think that on the other hand, for those countries where kavain and kava beverages are still used extensively, our analytical methods are of a practical value, and could help to answer and interpret forensic toxicological questions, such as the influence of kavain on driving ability or the synergetic effect of the combination of kavain with some other psychoactive drugs.

6 Summary

“Kava-Kava” is an intoxicating beverage made from the roots of Piper methysticum G. Forst. It is a widespread and since a long time well known drink on the islands of the South Pacific. For the effect of the kava drug certain ingredients of Piper methysticum, the so called kava-pyrones (kava-lactones), are made responsible. Kavain is one of the main kava-lactones representing about 5 to 12 % of the total amount of kava-lactones. Kavain is supposed to have similar to benzodiazepines an allosteric influence on the $GABA_A$ receptor-complex, through which the anxiolytic and antidepressant effect is explained. The fact that its central effects can generally lead to abuse, makes kavain an interesting subject for questions from the field of forensic toxicology. Thus the aim of this study was to develop suitable methods to detect and identify kavain and its metabolites and on this basis to gain new knowledge regarding the metabolism and pharmacokinetics of kavain. The available information from the literature on this topic is very incomplete.

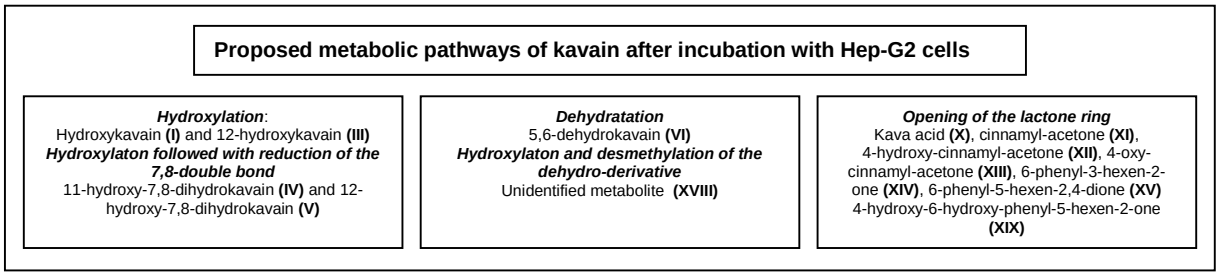
In an in-vitro study the kavain metabolism was first exemplary examined in Hep-G2 cells. After extraction (fluid-fluid extraction and SPE), kavain and its metabolites were identified and characterised by GC/MS (MSD HP 5970 (70 eV), GC HP 58790, column: HP-5 (i.D. 0,25 mm, 30 m); linearity for kavain (K) ranged between 20 to 5000 ng/ml (partly derivatised) and HPLC-DAD Waters Alliance with PDA 996, acetonitrile (31 % w/w) with phosphate-buffer pH = 2,3, flow 1,0 ml/min isocratic, column: LiChrospher 60 RP select B; linearity for kavain (K) from 5 to 100 ng/ml) and in some cases by LC/MS. To verify the suitability of the used methods, limits of detection, linearity and coefficients of variation were determined (GC/MS: for kavain (K), 12-hydroxykavain (III), 12-hydroxy-7,8-dihydrokavain (V) and 12-hydroxy-5,6-dehydrokavain (IX); HPLC-DAD: for kavain (K) and 12-hydroxykavain (III)). For the quantification HPLC-DAD proved to be suitable. For this, precision, reproducibility and repeatability were determined.

Unchanged kavain as well as 14 metabolites were extracted from the cell cultures after incubation with kavain. 12 of these metabolites are already known to be excreted via urine (human, rat). 4-Oxy-cinnamyl-acetone (XIII) is exclusively available in liver cell cultures, but not in blood or urine.

The detected metabolite pathways in the liver cells are hereafter: a) hydroxylation

(to I and III), hydroxylation with reduction of 7,8 double bonds (to give IV and V) and finally glucuronidation and sulfatation, b) dehydrogenation (to VI and after hydroxylation - to give IX and Ixa) and c) opening of the lactone ring, oxidation and decarboxylation at the side chain to give X, XI, XIII, XIV, XV and XIX. After enzymatic cleavage using ß-glucuronidase from E.coli a clear increase of IV is detectable, which is likely to be identical with 11-hydroxy-7,8-dihydrokavain according to the MS- and UV data.

Concrete hints regarding toxic effects could not be observed even after usage of high kavain amounts (up to 60 mg/ 200 ml culture media) and incubation times of up to 70 hours. Due to this, the metabolism studies were continued with the help of self-medication experiments. In a series of four experiments blood and urine samples were taken before and after oral uptake of 200 or 800 mg D,L-kavain and analysed.



Unchanged kavain could not be detected in urine. The kavain concentrations in blood and serum were very low compared to the administered dose (10 to 40 ng/ml, detectable 30 min. to about 4 hours after kavain uptake). It is obvious that kavain is quickly metabolised via the first pass effect.

Of the possible metabolic pathways, the hydroxylation at position C-12- of the phenyl ring and thus the formation of 12-hydroxykavain (III) plays a major part. The experiments revealed that the maximum concentration of III in blood is reached after 45 minutes. In serum 50 % of III were available as glucuronide, 12 % as sulfate, and 38 % in its free form (ratio of serum to blood is about 2:1). In urine only 1.4 % of III were excreted in its free form (maximum concentration after 2 hours). 15.3 % were found as glucuronide, and 83.3 % as sulfate.

Another possibility of the phenyl ring hydroxylation was that it has taken place at the non established position of the phenyl ring, this can produce the metabolite hydroxykavain (I). It is an important metabolite which is detected in blood after oral uptake of 800 mg kavain at concentrations of 30 to 50 ng/ml. The maximum was

reached after 2.5 hours. It was shown via enzymatic cleavage that this metabolite is not conjugated.

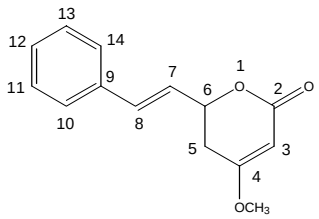
Dehydratation at the postion 5,6 of the lactone ring was the metabolism pathway to form 5,6-dehydrokavain (VI) which was detected in urine. It could be further metabolised to the unidentified metabolite (XVIII) by desmethylation at the C-4-position of the lactone ring. XVIII was detected in serum in concentrations between 38 and 84 ng/ml. The maximum concentrations were observed after 2 hours. In urine XVIII was still detectable after 24 hours. It was shown via enzymatic cleavage that this metabolite is partly conjugated as glucuronide or respectively sulfate.

In urine, though not in blood or serum, 12-hydroxy-7,8-dihydrokavain (V) could be detected as a representative of the 7,8-dihydro-derivates. It is remarkable that this metabolite is eliminated by the urine just after 5 hours from oral uptake and exclusively in its conjugated form (glucuronide:sulfate = 2:1)

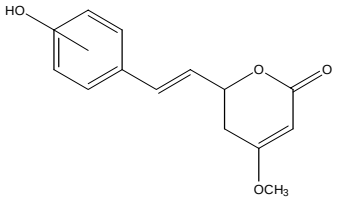
The last degradation pathway which was observed in the liver cell experiment could be detected as well, the metabolite 6-phenyl-5-hexene-2,4-dione (XV) was detected in urine by GC/MS as well as LC/MS.

For the metabolite 12-hydroxykavain (III) and its conjugates (glucuronide, sulfate) additional kinetic parameters (rate constant of terminal slope, plasma elimination half life, AUC, volume of distribution as well as renal clearance) were determined.

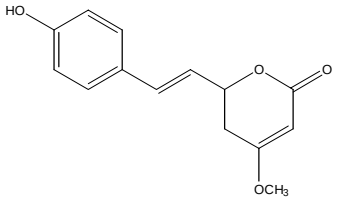
The presented results revealed new insights regarding the kavain metabolism. The degradation pathways of kavain proved to be applicable from the in-vitro model to humans by evidence of the metabolites. The methods used for identification and quantification of kavain and its metabolites could be validated and thus comply with the requirements of forensic toxicology. In the field of forensic toxicology and traffic medicine, it was necessary to have a validated method for the determination of kavain and its metabolites in different materials to give an expert opinion in cases of misuse or abuse of this drug. Our experiences show that there is a big interest in such methods in countries with widespread kava consumption. Some aspects of the kavain metabolism are therefore resolved, though further research is necessary to fully comprehend the pharmacokinetics and -dynamics of the kava-lactones.



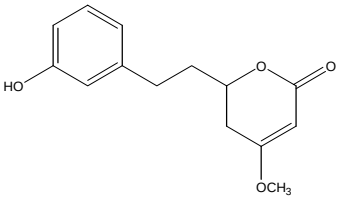
Kavain (K)



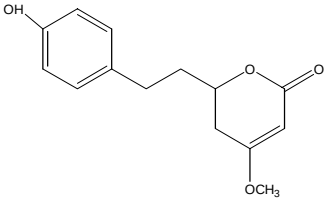
Hydroxykavain (I)



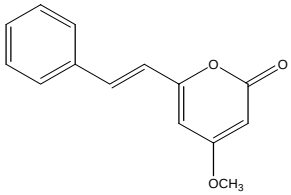
12-Hydroxykavain (III)



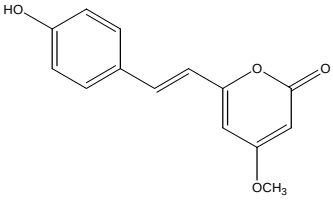
11-Hydroxy-7,8-dihydrokavain (IV)



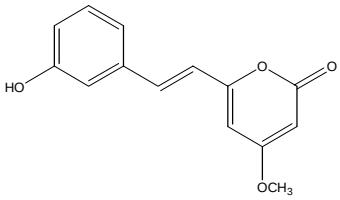
12-Hydroxy-7,8-dihydrokavain (V)



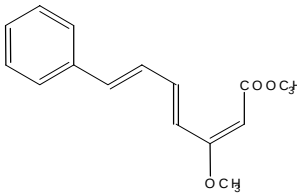
5,6-Dehydrokavain (VI)



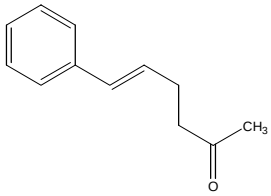
12-Hydroxy-5,6-dehydrokavain (IX)



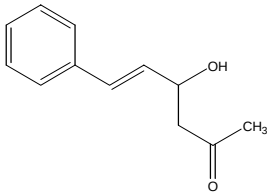
11-Hydroxy-5,6-dehydrokavain (IXa)



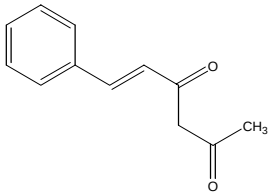
Kava acid methyl ester (X)



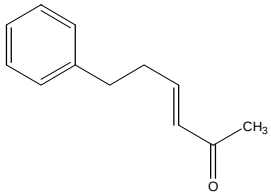
Cinnamyl-acetone (XI)



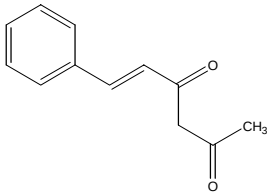
4-Hydroxy-cinnamyl-acetone (XII)



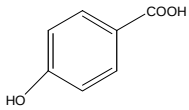
4-Oxy-cinnamyl-acetone (XIII)



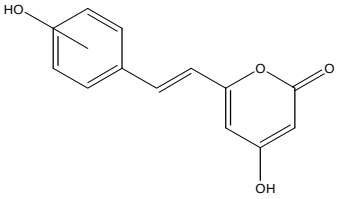
6-Phenyl-3-hexen-2-one (XIV)



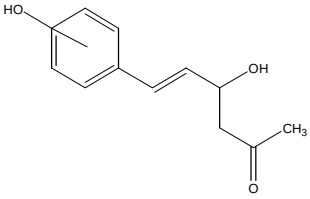
6-Phenyl-5-hexen-2,4-dione (XV)



p-Hydroxy-benzoic acid (XVI)



Unidentified metabolite (XVIII)



4-Hydroxy-6-hydroxyphenyl-5-hexen-2-one (XIX)

Figure 56: Chemical structures of D,L-kavain and its metabolites detected in this study

7 Zusammenfassung

„Kawa-Kawa“ ist ein aus der Wurzel von Piper methysticum G. Forster hergestelltes, auf den Südseeinseln lange bekanntes und weit verbreitetes, berauschend wirkendes Getränk. Für die Wirksamkeit der Kava-Droge werden bestimmte Inhaltsstoffe von Piper methysticum, die sogenannten Kavapyrone (Kavalactone), verantwortlich gemacht. Mit ca. 5 bis 12 % Anteil ist Kavain eines der Haupt-Kavalactone. Kavain soll, ähnlich wie die Benzodiazepine, einen allosterischen Effekt auf den $GABA_A$-Rezeptor haben, wodurch seine anxiolytische und antidepressive Wirkung erklärt wird. Da seine zentralen Wirkungen grundsätzlich zu einem Missbrauch führen können, wird es auch für Fragestellungen aus der forensischen Toxikologie interessant. Ziel dieser Arbeit war es daher, geeignete Verfahren zum Nachweis und zur Identifikation von Kavain und seinen Metaboliten zu entwickeln und auf dieser Basis neue Erkenntnisse zum Metabolismus und zur Pharmakokinetik von Kavain zu erlangen. Die Literatur gibt hierzu nur sehr unvollständig Auskunft.

In einer in-vitro-Studie wurde modellmäßig zuerst der Metabolismus von Kavain in Hep-G2-Zellen untersucht. Nach Extraktion (Flüssig-Flüssig-Extraktion und SPE) wurden Kavain und seine Metaboliten mittels GC/MS (MSD HP 5970 (70 eV), GC HP 58790, Säule: HP-5 (i.D. 0,25 mm, 30 m); Linearität für Kavain (K) im Bereich von 20 bis 5000 ng/mL (hierfür teilweise derivatisiert) und HPLC/DAD Waters Alliance mit PDA 996, Acetonitril (31 % w/w) mit Phosphatpuffer pH = 2,3, Flow 1,0 mL/min isokratisch, Säule: LiChrospher 60 RP select B; Linearität für Kavain (K) von 5 bis 100 ng/mL) sowie in einigen Fällen mit LC/MS identifiziert und charakterisiert. Um die Eignung der angewendeten Methoden sicherzustellen, wurden Nachweisgrenzen, Linearitätsbereiche und Variationskoeffizienten bestimmt (GC/MS: für Kavain (K), 12-Hydroxykavain (III), 12-Hydroxy-7,8-dihydrokavain (V) und 12-Hydroxy-5,6-dehydrokavain (IX); HPLC: für Kavain (K) und 12-Hydroxykavain (III)). Für die Quantifizierung erwies sich die HPLC mit DAD als besonders geeignet. Hierfür wurden auch Präzision, Reproduzierbarkeit und Wiederfindung bestimmt.

Aus den Zellkulturen wurden nach Inkubation mit Kavain unverändertes Kavain sowie 14 Metaboliten extrahiert. Von 12 dieser Metaboliten ist bereits bekannt, dass sie mit dem Urin ausgeschieden werden (Mensch, Ratte). 4-Oxy-cinnamyl-aceton (XIII) und der bisher unbekannte Metabolit XVIII wurden erstmals in Hepatozellen

nachgewiesen. Bei späteren Untersuchungen zeigte sich, dass XIII ausschließlich in den Leberzellkulturen aufzufinden war.

In den Leberzellen nachgewiesene Stoffwechselwege sind danach a) Hydroxylierung (zu I, III, IV, V und zu den 7,8-Dihydroderivaten IV und V) und schließlich Glucuronidierung (IV), b) Dehydrierung (zu VI und nach Hydroxylierung – s.o. - zu XVIII) sowie c) Öffnung des Lactonringes und Oxidation an der Seitenkette (X, XI, XIII, XIV, XV und XIX). Nach Spaltung mit ß-Glucuronidase ist ein deutlicher Anstieg von IV festzustellen, das nach den MS- und UV-Daten sehr wahrscheinlich mit 11-Hydroxy-7,8-dihydrokavain identisch ist.

Konkrete Hinweise auf toxische Effekte konnten auch bei Einsatz hoher Kavain-Mengen (bis 60 mg/ 200 mL Kulturmedium) und Inkubationszeiten bis zu 70 Stunden nicht beobachtet werden. Daher wurden die Metabolismusuntersuchungen im Selbstversuch fortgesetzt. In vier Versuchsreihen wurden vor und nach oraler Einnahme von 200 oder 800 mg D,L-Kavain Blut und Urin abgenommen und untersucht.

Unverändertes Kavain war im Urin nicht nachweisbar. Die in Blut und Serum aufgefundenen Kavain-Konzentrationen waren im Vergleich zur applizierten Dosis sehr niedrig (10 bis 40 ng/mL, nachweisbar 30 min. bis ca. 4 Stunden nach Kavaineinnahme). Kavain wird offensichtlich sehr schnell über den First Pass-Effekt metabolisiert. Unter den möglichen Stoffwechselwegen spielt die Hydroxylierung an der C-12-Position des Phenylrings und damit die Bildung von 12-Hydroxykavain (III) eine Hauptrolle. Die Versuche ergaben, dass die maximale Konzentration von III im Blut nach 45 Minuten erreicht wird. Im Serum lagen 50 % von III als Glucuronid, 12 % als Sulfat und 38 % in freier Form vor (Verhältnis Serum zu Blut ca. 2:1). Im Urin wurden nur 1,4 % III in freier Form ausgeschieden (maximale Konzentrationen nach 2 Stunden). 15,3 % waren glucuronidiert, und 83,3 % lagen als Sulfat vor.

Erstmals konnte gezeigt werden, dass Hydroxykavain (I) einen wichtigen, im Blut nachweisbaren Metaboliten darstellt. Im Blut wurde es nach oraler Gabe von 800 mg Kavain in Konzentrationen von 30 bis 50 ng/mL nachgewiesen. Das Maximum wurde nach 2,5 Stunden erreicht. Durch enzymatische Spaltung wurde gezeigt, dass dieser Metabolit nicht glucuronidiert oder sulfatiert vorliegt.

5,6-Dehydrokavain (VI) wird durch Desmethylierung in C-4-Position des Lactonringes weiter zu dem unbekannten Metabolit XVIII metabolisiert. XVIII wurde im Serum in

Konzentrationen zwischen 38 und 84 ng/mL aufgefunden, die maximalen Konzentrationen wurden nach 2 Stunden beobachtet. Im Urin war XVIII auch noch nach 24 Stunden nachweisbar. Durch enzymatische Spaltung wurde gezeigt, dass auch dieser Metabolit teilweise glucuronidiert bzw. sulfatiert vorliegt.

Im Urin, jedoch nicht im Blut bzw. Serum, konnte weiterhin als Vertreter der 7,8-Dihydro-Derivate 12-Hydroxy-7,8-dihydrokavain (V) nachgewiesen werden. Bemerkenswert ist, dass dieser Metabolit erst 5 Stunden nach der oralen Aufnahme und ausschließlich in konjugierter Form (Glucuronid : Sulfat = 2 : 1) über den Urin eliminiert wird.

Auch der letzte bei dem Leberzellenversuch nachgewiesene Abbauweg (Öffnung des Lactonringes) konnte gefunden werden. Im Urin wurde der Metabolit 6-Phenyl-5-hexen-2,4-dion (XV) sowohl mit GC/MS als auch mit LC/MS nachgewiesen.

Für den Hauptmetaboliten 12-Hydroxykavain (III) und seine Konjugate (Glucuronid, Sulfat) wurden zusätzlich kinetische Parameter (Geschwindigkeitskonstante, Plasmaeliminations-Halbwertszeit, AUC, Verteilungsvolumen sowie renale Clearance) bestimmt.

Mit den hier vorgestellten Ergebnissen haben sich für die Kava-Forschung, die insbesondere zur Hepatotoxizität von Kavalactonen noch Fragen beantworten muss, wichtige neue Erkenntnisse hinsichtlich des Kavain-Metabolismus ergeben. Die Abbauwege von Kavain erwiesen sich als aus dem in-vitro-Modell auf den Menschen übertragbar und wurden über die Metaboliten abgesichert. Die zur Identifizierung und zur Quantifizierung von Kavain und seinen Metaboliten eingesetzten Methoden haben sich zudem als validierbar erwiesen und entsprechen damit den Erfordernissen der forensischen Toxikologie. Bei forensischen und verkehrsmedizinischen Fragestellungen muss der Nachweis von Kavain und damit einer auf dem Gebrauch der Kava-Droge basierenden Wirkung in Körperflüssigkeiten und Organen geführt werden können. Nach unseren Erfahrungen besteht in den Ländern, in denen der Kava-Konsum verbreitet ist, ein großes Interesse an solchen Methoden.

Teilaspekte des Kavain-Metabolismus sind damit geklärt, es werden aber auch weiterhin noch Forschungsanstrengungen notwendig sein, um Pharmakokinetik und –dynamik der Kava-Wirkstoffe vollständig zu verstehen.

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1978-1981	Elemantary School (Derna, Libya)
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1984-1989	Faculty of Pharmacy (Al-Fateh-University of Medical Sciences Tripoli, Libya)
1995 (5 month)	Language Course at Goethe-Institute, Bonn

Degrees

1984	Bachelor of Science
1989	B. SC. Pharm.
2000	Diploma in Forensic Toxicology of the German Society of Legal Medicine

Career

1989-1991	Pharmacist in Al-Wahda Hospital (Derna, Libya)
1990-1995	Director of a private pharmacy (Derna, Libya)
1992-1994	Military Service (24 months) in Tobruk, Libya

1994-1995	Director of the central laboratory of the Al-Wahda Hospital (Derna, Libya)
Since 1996	Guest Scientist at the Institute of Legal Medicine Düsseldorf as part of a scholarship till March 2000 for a post graduate study in Forensic Toxicology and a PhD degree in pharmacy
Since 1997	Participation in the AK Extraktion der GTFCh
Since 2000	Scientific Assistant at the Institute of Legal Medicine Düsseldorf

Memberships

Gesellschaft für Toxikologische und Forensische Chemie (GTFCh)

The International Association of Forensic Toxicologists (TIAFT)