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QUATERNARY BENZO[*c*]PHENANTHRIDINES SANGUINARINE AND CHELERYTHRINE: A REVIEW OF INVESTIGATIONS FROM CHEMICAL AND BIOLOGICAL STUDIES

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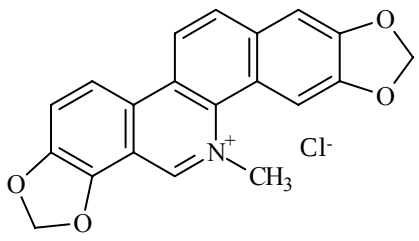
Abstract – Sanguinarine and chelerythrine are intensively studied biologically active alkaloids for their potentially useful medicinal properties, such as antimicrobial, antiinflammatory, and antitumoral activities. This article aims to review critically recent literature published on the chemical behavior, synthesis, analytical methods and biotransformation of both alkaloids.

INTRODUCTION

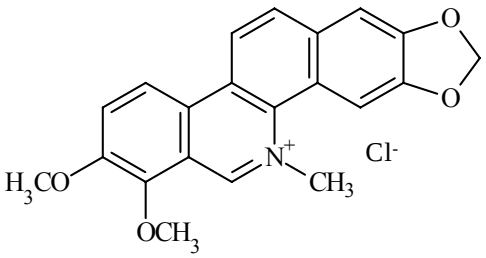
Medicinal plants containing quaternary benzo[c]phenanthridine alkaloids (QBA), sanguinarine (SA) and chelerythrine (CHE) - *Sanguinaria canadensis*, *Chelidonium majus* and *Macleaya cordata* (Papaveraceae) were in the past utilized in traditional medicine long before the isolation of pure alkaloids.[1] SA and CHE are the subject of sustained practical and research interests owing to their pronounced widespread biological activities.[2, 3] Both alkaloids display antimicrobial, anti-inflammatory, adrenolytic, sympatholytic, cytostatic and local anesthetic effects.[4] The powdered rhizomes and roots of *S. canadensis* are the active components in the weight gain stimulant for farm animals SANGROVIT®.[5] QBA fractions from *S. canadensis* (SANGUINARIA) and *M. cordata* (SANGUIRITRIN) are used in

toothpastes and mouthwashes as antiplaque agents. SANGUIRITRIN is applied as an antifungal and anti-inflammatory preparation in Russia.[2]

SA and CHE interconvert between the cationic *vs* neutral form, i.e. pseudobase or 6-hydroxy-5,6-dihydroderivative.[6] They penetrate across the cell membrane in the hydrophobic pseudobase form acting as the pro-drugs and convert into the active cationic form once inside the cell. The C(6)=N^+(5) iminium bond of QBA is susceptible to a nucleophilic attack and plays a key role in inhibition of SH-proteins.[7] SA as well as QBA-containing extracts exhibit a very low acute oral toxicity. In subchronic studies, a minor evidence of treatment-related toxicity of QBA (doses > 30 mg/kg/day; rat, monkey) was reported.[8] The available data do not predict a health hazard to humans and farm animals when QBA-containing oral hygiene products and feed additives are used.[4] A well-known toxic effect attributed to QBA is the epidemic dropsy syndrome, which is associated with the consumption of plant oils contaminated by alkaloids of *Argemone mexicana.*[9] Damm [10] has reported that the long-term use of oral products containing sanguinaria appears to be associated with an increased prevalence of leukoplakia of maxillary vestibule mucous membrane.



SANGUINARINE



CHELERYTHRINE

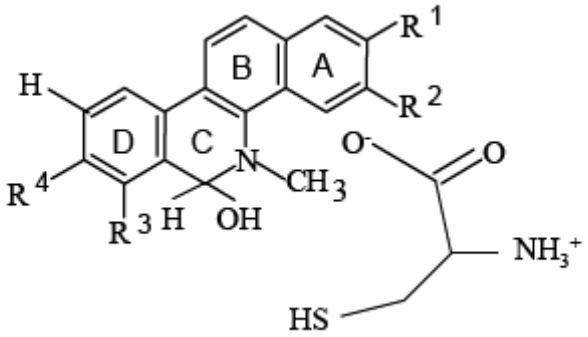
CHEMICAL BEHAVIOR OF SANGUINARINE AND CHELERYTHRINE

The double bond C(6)=N^+(5) (iminium bond) of SA/CHE is sensitive to attack by nucleophiles. For hydroxide ion as a nucleophile, a pH dependent equilibrium between the quaternary cation and the pseudobase form is typical of QBA. Involving protolysis in water the process may be formulated as a reversible acidobasic equilibrium $Q^+ + H_2O \rightleftharpoons QOH + H^+$ with an equilibrium constant $K_{R+} = [H^+][QOH]/[Q^+]$. Constants pK_{R+} that characterize equilibrium between charged and uncharged forms of SA and CHE range between 7 and 9.[11] Thus, at physiological pH 7.4, both charged and uncharged forms of the alkaloids exist in aqueous solutions and in the blood. SA and CHE reportedly interact with nucleophilic SH- groups of simple organic compounds in a 1:1 ratio.[7] An analogous interaction is

suggested with SH-enzymes [7] and with human serum albumin [12] in which the one free SH-group is expected to be the interaction point. The charged (the iminium) forms of SA and CHE have been indicated as the forms interacting chemically with nucleophiles including mercapto ones [7]; the latter was deduced from static photometric measurements. In contrast, the pseudobase form of SA was denoted as the form interacting with human serum albumin.[12]

Our electrophoretic investigation into the type of interaction between SA or CHE and L-cysteine or mercaptoethanol evidenced that interaction of these alkaloids with both mercapto compounds was not a chemical reaction but a complexation. The formed complex based on non-bonding intermolecular interactions is kinetically labile. Measurements at various pH revealed that only uncharged forms of these alkaloids complex with simple mercapto compounds.[13, 14] L-Cysteine is uncharged in the pH range 5 – 7.4. Anionic migration of the complex formed by uncharged SA or CHE and mercaptoethanol at pH 7.4, and at high concentrations of mercaptoethanol in the background electrolyte was observed. This migration evidences that an anion, which may be supplied only from the background electrolyte, participates in the formation of the complex between SA or CHE and mercaptoethanol. The absence of an analogical effect in experiments with L-cysteine may be explained by the participation of the negative charge of L-cysteine in the complexation. The stability of complexes of SA and CHE with mercaptoethanol and L-cysteine depends on both buffer cation and buffer anion. Constants corrected for the abundance of the uncharged form of these alkaloids therefore differ markedly depending on the buffer composition. For example, stability of complexes of SA with L-cysteine was from 8,400 to 18,000 $mol.l^{-1}$, and that of CHE was from 21,500 to 30,800 $mol.l^{-1}$ in the pH range.[13, 14] The conclusion from experiments with mercaptoethanol was that some participation of the negative charge is necessary for the non-covalent binding of uncharged SA or CHE with the SH-group. If L-cysteine is the ligand, its carboxylic group may supply the necessary negative charge and the resulting complex remains therefore outwardly uncharged. If the negatively charged group is absent from the ligand, *e.g.*, in mercaptoethanol, the necessary negative charge may be supplied only from the solution. In this case, the resulting complex is negatively charged. The simple 1:1 interaction scheme therefore, holds only for mercapto compounds bearing negatively charged groups. Identical results were obtained from the investigation of the complexation of SA and CHE with human serum albumin. Constants corrected for the abundance of the interacting uncharged form of these alkaloids were 332,000±38,400 and 2,970,000±360,000 $mol.l^{-1}$ for SA and CHE, respectively.

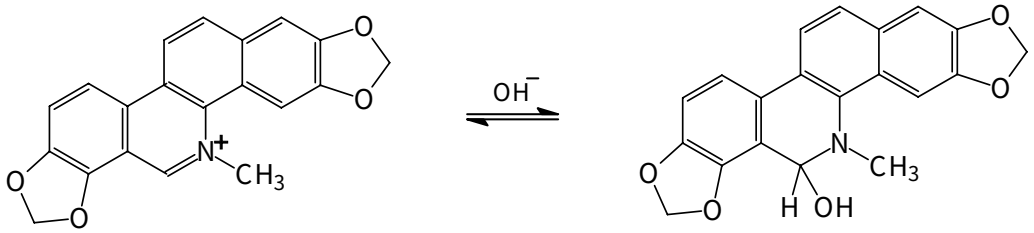
Finally, non bonding interactions between benzophenanthridines and SH-group were reported (see below).[13, 14]



STRUCTURE STUDIES OF SANGUINARINE AND CHELERYTHRINE

Quaternary cationic structures of sanguinarine and chelerythrine have been well known since 1931. They are aromatic systems, planar, and as such not too interesting from structural aspect. ^{1}H, ^{13}C and ^{15}N NMR spectra of quaternary benzo[*c*]phenanthridine alkaloids including sanguinarine and chelerythrine were extensively studied by Marek *et al*.[15] The assignment of signals in the ^{1}H and ^{13}C spectra was obtained from 2D NOE and gradient-enhanced single-quantum multiple bond correlation (GSQMBC) experiments. To support the assignment, chemical shielding was calculated after *ab initio* geometry optimization. The NMR characteristics related to the skeleton substitution pattern were described.

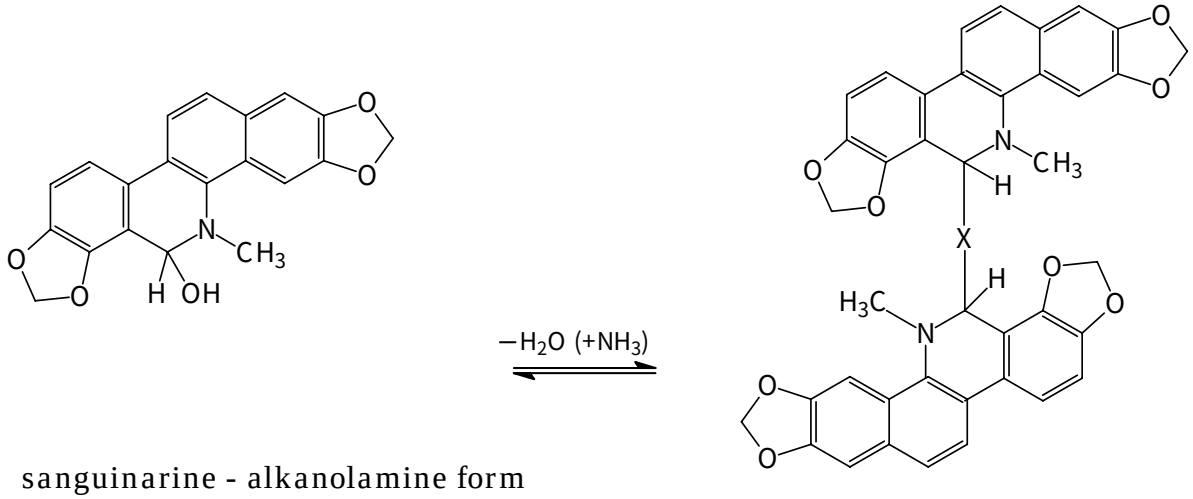
Knowledge of structures formed from quaternary cations upon alkalization dates back only to the 1990'. As mentioned at the beginning of this review, sanguinarine and chelerythrine quaternary cations easily add hydroxide ions in a nucleophilic attack on the reactive $C=N^{+}$ bond under conversion into "free bases", 6-hydroxy-5,6-dihydroderivatives, also termed alkanolamines or, in a rather obsolete way, pseudobases. 6-Hydroxy-5,6-dihydroderivatives are generally considered being the molecule that penetrates cell membranes much easier that the corresponding cation and hence plays an important role in QBA biological activity.



sanguinarine cation sanguinarine „free base" (alkanolamine)

QBA free bases have been systematically studied by Sklenář group.[16, 17] They found that 6-hydroxy-5,6-dihydroderivatives of sanguinarine and chelerythrine are unstable and are easily converted into dimeric ethers (bimolecular aminoacetals) (in aqueous K_2CO_3) or dimeric amines (in aqueous NH_3). Bimolecular products were isolated directly from aqueous solutions or could be extracted and crystallized

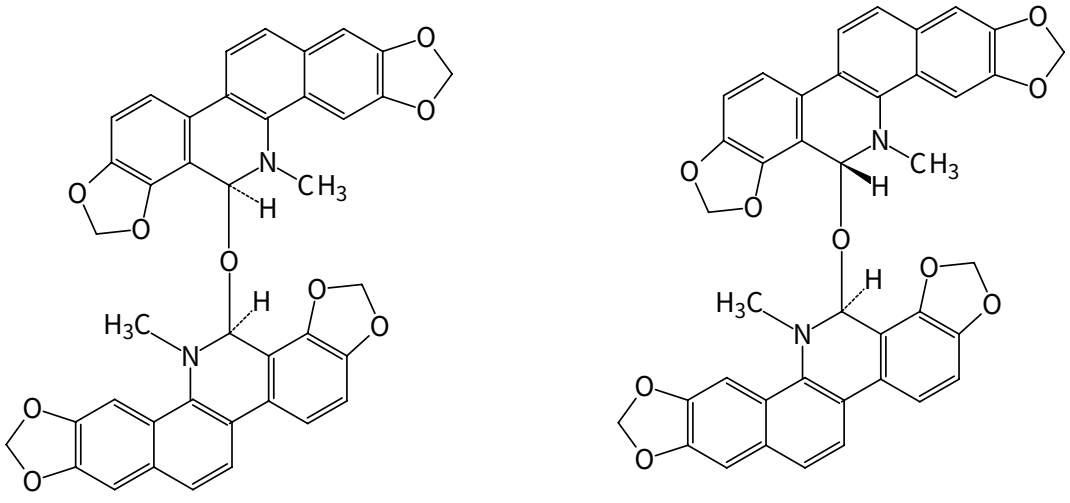
from diethyl ether. They are not very stable, in DMSO-d_6 or $CDCl_3$ containing residual water they hydrolyze to (monomeric) alkanolamines.



sanguinarine - alkanolamine form

bis(dihydrosanguinarinyl) ether X =O
bis(dihydrosanguinarinyl)amine X = NH

Toušek et al. studied configurations and conformations of bimolecular aminoacetals of sanguinarine ad chelerythrine by NMR spectroscopy, quantum chemical calculations and X-Ray analysis.[18] Sanguinarine free base dissolved in $CDCl_3$ contains a mixture of alkanolamine and both diastereomers of the bimolecular aminoacetal in the ratio of appr. 95 : 5. X-Ray diffraction analysis shows that in bis(dihydrosanguinarinyl)ether the two benzo[*c*]phenanthridine units are almost perpendicular; their best planes make an angle of 74.2°. The shape of the molecule resembles the 'L' letter. Geometry of the molecule as well as chemical shifts of ^1H and ^{13}C atoms was also obtained from quantum chemical calculations. The highest stability was found for the diastereomer (6*R*, 6´*R* and 6*S*, 6´*S*) with population of 96.4%. The calculated structure was in good agreement with the conclusions drawn from NMR and X-Ray analyses.



6*S*, 6´*S* 6*R*, 6´*S*

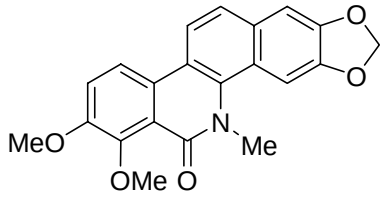
bis(dihydrosanguinarinyl) ether diastereomers

6-Hydroxy-5,6-dihydroderivatives (alkanolamines, pseudobases) cannot be isolated. They exist as the only product of alkalization of sanguinarine and chelerythrine by aqueous Na_2CO_3 in DMSO-d_6 solution. In less polar media (C_6D_6, $CDCl_3$) the formation of bimolecular aminoacetals is preferred; in water-containing nonpolar solvents there is equilibrium alkanolamine – bimolecular aminoacetal. It is assumed that the free bases of sanguinarine and chelerythrine adopt the (monomeric) alkanolamine structures in biological systems in the presence of water and ions.[19]

We studied the behavior of sanguinarine with nucleophilic agents such as mercaptoethanol, cysteine or albumin in phosphate buffers by electromigration methods.[13, 14] The simple 1 : 1 interaction (see the part on chemical behavior above) means the occurrence of (monomeric) alkanolamine form, which is not in contradiction to the above-mentioned assumption of Sečkářová *et al*.[19]

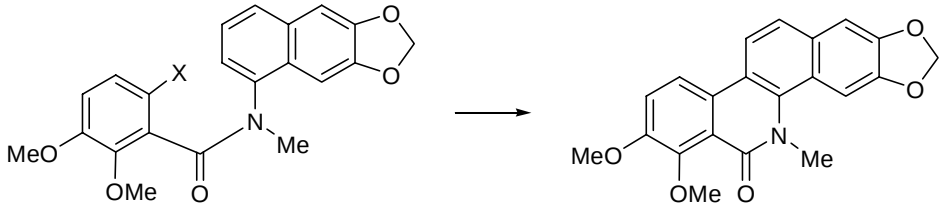
SYNTHESIS OF CHELERYTHRINE AND SANGUINARINE

Synthetic procedures for the preparation of benzo[*c*]phenanthridine alkaloids remain less studied than extraction methods. A problem with synthesis is the necessity for multistep procedures with low overall yields and the lack of general method of preparation. Advanced methods for total synthesis of chelerythrine published over the last five years use the analogue – oxychelerythrine **(1)** as a convenient intermediate. Its conversion to the desired final product is then based on a standard and well-known reaction.[20]



(1)

One promising method, which appears to be both accurate and versatile for the preparation of a benzo[*c*]phenanthridine alkaloid such as chelerythrine is palladium-assisted aryl-aryl coupling reaction of 2-halo-*N*-arylbenzamides.[21] The crucial coupling step of haloamides **(2)** with $Pd(OAc)_2$, PPh_3 or P(*o*-tol)$_3$ and Ag_2CO_3 in DMF afforded oxychelerythrine **(1)** in a yield of 85-96 %.



2a X = I
2b X = Br

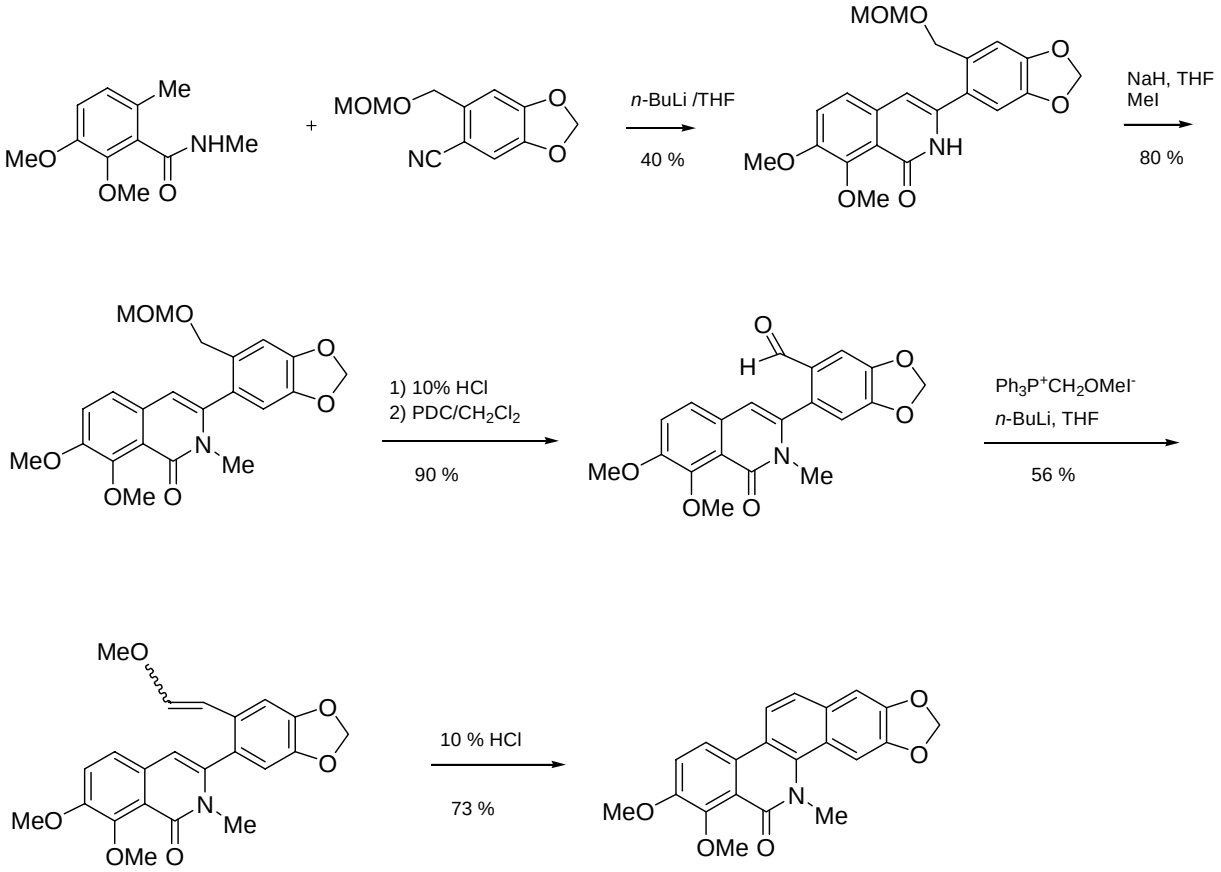
1

The halogen X in compound **(2)** can be replaced by the trifluoromethanesulfonyloxy group (OTf). Then the combination of palladium acetate $Pd(OAc)_2$, 1,3-bis(diphenylphosphino)propane (DPPP) and tributylphosphine Bu_3P is successfully used as a catalyst mixture to give oxychelerythrine **(1)** in high yield.[22] The choice of base plays a crucial role in yields and reaction time. When DBU is used the reaction takes 1 hr and gives a product yield of 63 %.[23] The same yield can be achieved with the use of Ag_2CO_3 after 4 hrs. When $(i\text{-}Pr)_2NEt$ is used as a base the product obtained is 81 % yield over 30 min.[24]

The abovementioned combination of catalyst and bases can also be used for a coupling reaction of halogenederivatives **(2)** with reaction time 15-30 min in yields 79-95 %.[22]

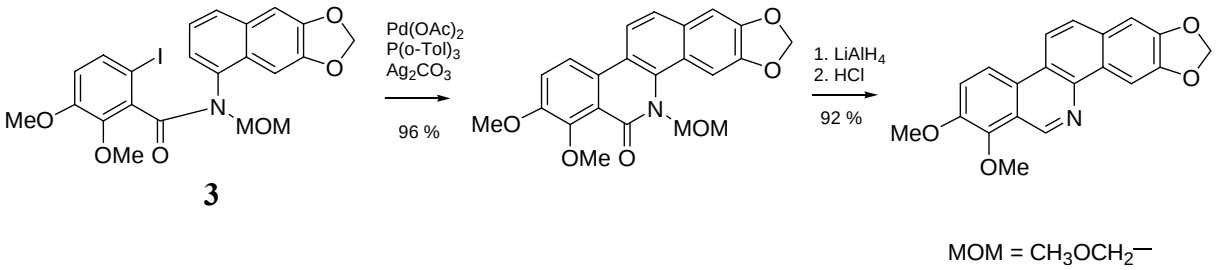
Although the catalyzed reaction using a mixture of $Pd(OAc)_2$ and Bu_3P has been reported,[25] the procedure was not useful for coupling reactions between triflate and arenes with oxygen functionalities.

Another strategy for oxychelerythrine synthesis is based on lithiated toluamide-benzonitrile cycloaddition.[26] This six-step synthesis seems to be also very efficient and includes easily preparable starting material.

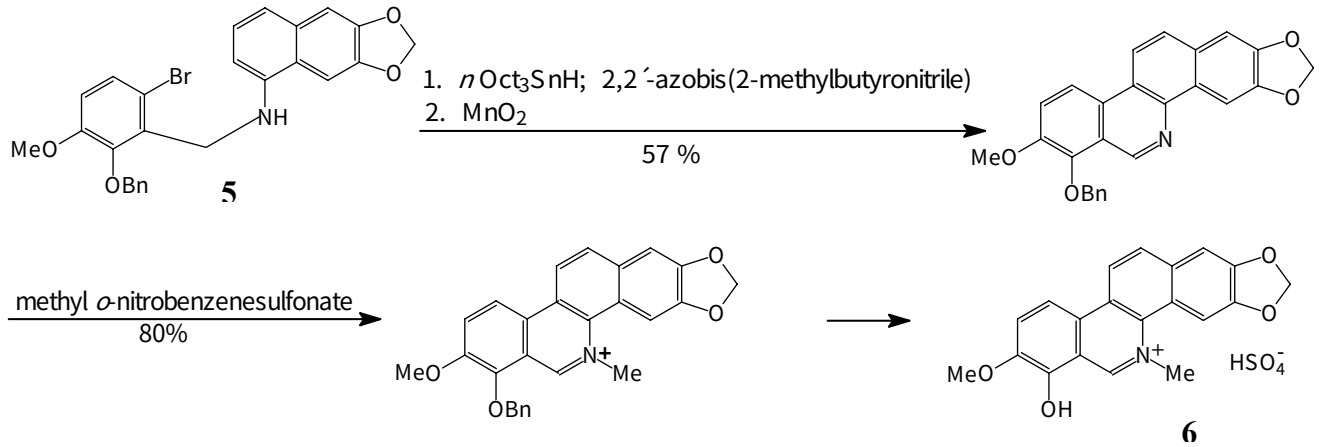


Scheme

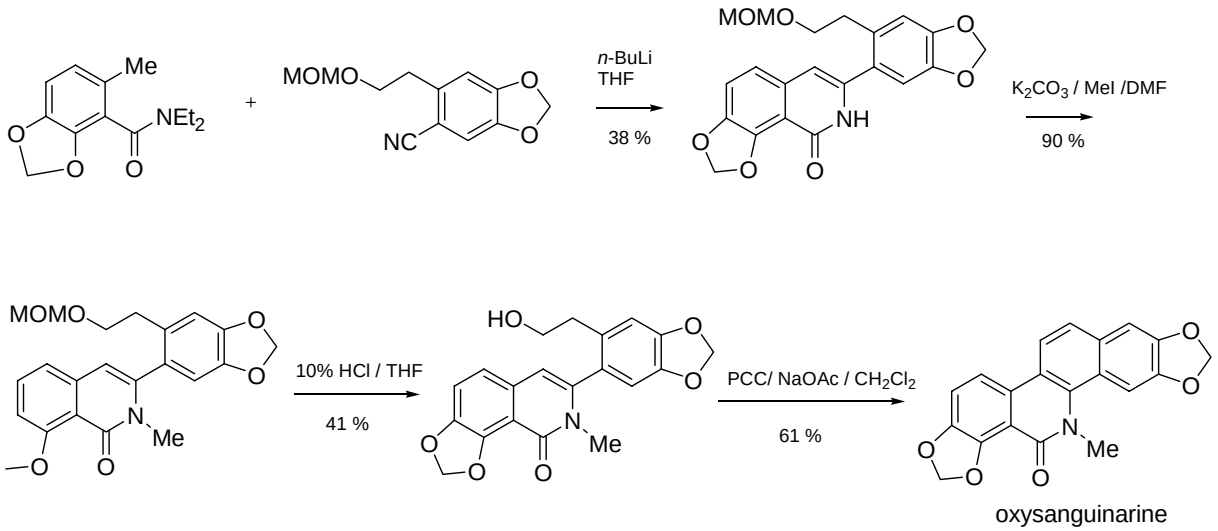
The coupling reaction was also successfully used for synthesis of norchelerythrine [27] another good precursor for chelerythrine synthesis, methylation of which has been described previously.[28] This coupling reaction uses iodo-*N*-methyloxymethylbenzanilide derivative **(3)** as a reactant and $Pd(OAc)_2$ as a catalyst. The best ligand/base combination $P(o\text{-}Tol)_3$ and Ag_2CO_3 was found.



7-Hydroxy analogue of chelerythrine (NK 109, **(6)**) is a compound with interesting antitumor activity. Its synthesis uses benzyl-protected bromoamine **(5)** as a key precursor. The benzo[*c*]phenanthridine ring is obtained by its radical cyclization followed by oxidative aromatization. N-methylation, rather difficult in this case, was achieved with methyl o-nitrobenzenesulfonate.[29]



Sanguinarine synthesis was not studied so intensively in last five years. Only one paper describing the lithiated toluamide-benzonitrile cycloaddition leading to oxysanguinarine was published.[30]



ANALYTICAL METHODS

Today many analytical procedures are described for the determination of benzo[*c*]phenanthridine alkaloids in biological materials (plant extracts, animal tissues, *etc*.), pharmacological and veterinary preparations and for studies of their biotransformations. They involve paper chromatography [31] and classical [32-34] and high performance [35-39] thin-layer chromatography, gas chromatography with mass spectrometric detection,[33, 40] high performance liquid chromatography utilizing UV-VIS spectrophotometric, fluorimetric [41-43] and mass spectrometric [44-46] detection systems and electromigration methods, including isotachophoresis [47, 48] and capillary zone electrophoresis.[41, 42, 49]

Isolation, purification and pre-separation

Isolation and pre-concentration of a substance of interest from a biological matrix is the most crucial part of an analytical procedure, especially at very low (sub-micromolar) concentrations. Besides isolation and pre-concentration steps, it is of great importance to obtain a very clean extract suitable for final analyses. This is fairly easily accomplished in the case of biological materials rich in the substance(s) of interest. Several rapid and reliable solid-phase extraction (SPE) procedures for the isolation of alkaloids from plants have been established. Usually methanol (MeOH), ethanol or acetonitrile (ACN) acidified with HCl, acetic or formic acids are successfully employed for plant materials, feed, faeces, and as well as cells in in-vitro experiments with recoveries 80 – 95%, except of 65% recoveries for SA in cells.

Microwave-assisted extraction (MAE) was found to be the most effective method of the examined and compared methods (maceration, MAE, ultrasound-assisted extraction - UAE and percolation) capable of yielding SA and CHE with a short extraction time.[50] Dried and powdered plant samples could be also extracted with acetic acid under UAE.[51] Application of an ion-pairing agent, 1-heptanesulfonic acid, for

isolation of QBA from acidified methanolic solutions of commercial tinctures of *Chelidonium majus* L. was also described.[52]

After centrifugation, the supernatant is loaded on SPE cartridge and repeatedly washed with acidified water:organic solvent mixture (0.1 M HCl and methanol, ACN) or methanolic HEPES solution.[52] Remaining alkaloids are eluted with an aqueous alkaline methanol,[50] water:organic solvent mixture or HEPES in 95% methanol and concentrated under nitrogen stream at reduced pressure before HPLC analysis. Bioassay guided fractionation led to the isolation of both, sanguinarine and chelerythrine, from the roots of *S. canadensis* crude methanolic extracts.[53]

Column chromatography on Merck silica gel 60 was also used for pre-separation of alkaloids from *Bocconia arborea* with chloroform:MeOH (5:1, v/v) into seven fractions. The fractions were analysed by GC/MS.[40] Two groups, tertiary and quaternary alkaloids (namely SA, CHE, DHSA, DHCHE, chelilutine, chelirubine, and berberine), have been separated from *Chelidonium majus* L. on a classical silica column by use of methanol in the acetate buffer of pH 6.0.[37] The retention of alkaloids in such a system depends both on the concentration of methanol and on the pH of the mobile phase. Gradient elution with increasing concentration of methanol allowed separation of the QBA further into fractions containing 2 or 3 alkaloids. These fractions were then separated by micropreparative zone TLC to obtain SA and CHE of high purity.

Complications appeared during isolation of the alkaloids from animal tissues, especially when intact tissues with multiple cell types are present. Alkaloids proceed to a more or less strong interactions with biomacromolecules, such as proteins, peptides, nucleic acids *etc*., and even with several low-molecular substances.[13, 54] Thus the interactions are responsible for retention of alkaloids in sample matrices based on animal tissues. Adjusting the content of organic solvent, pH and ionic strength of the aqueous medium is a classical approach to release alkaloids from the bonds with the biomacromolecules into an extractant.

For HPLC, CE and HPLC/ESI-MS analysis of SA and DHSA,[41, 44] methanolic solutions or plasma specimens diluted with the ion-pairing agent, 10 mM 1-heptanesulfonic acid, were loaded onto the conditioned C18 SPE cartridges, washed with the HEPES solution and the retained QBA were eluted with 10 mM HEPES in 95% methanol with recoveries cca. 50%. After evaporation of the eluate and dissolution in the mobile phase, the samples were ultra-filtered and applied onto an HPLC column. The liver homogenate in 10 mM HEPES in 95% acetonitrile was vortexed vigorously, sonicated, and centrifuged. The supernatant was evaporated under N_2 at 50 ºC, dissolved in methanol and applied onto an HPLC column. Urine diluted 1:1 with mobile phase was directly applied onto an HPLC column undergoing the same procedure.[41] The recoveries were significantly lower (15-20% for porcine liver, gingiva and stomach).

Supercritical fluid extraction (SFE) was successful in extractions of non-polar biologically active compounds from plant materials. A high flow-rate modification of the method is successful even for the extraction of polar compounds from complex animal matrices.[55] In addition, complex matrices contain molecules capable to act as entrainers that further enhance the method recovery. For HPLC/ESI-MS, highly rapid, reliable, effective and reproducible extraction methods have been compared. The supercritical fluid extraction (SFE) at 35 °C and 10 MPa (15 MPa for real samples) was found to be the most effective method of the tested ones (maceration, microwave-assisted extraction - MAE, ultrasound-assisted extraction – UAE, and modified Soxhlet extraction) capable of yielding SA and CHE and their dihydroderivatives and metabolites from animal tissues [44, 55] with acceptable recoveries (95 % and 81 %, resp.) using near-critical CO_2 modified with aqueous methanol.

Separation, identification and determination

Chromatographic methods - planar chromatography: High-performance thin-layer chromatographic (HPTLC) quantification of SA as an index of argemone oil adulteration [35] was achieved by densitometric scanning of the plate in fluorescence/reflectance mode (no elaborate extraction procedure is needed). DHSA was determined after its conversion to SA by UV (366 nm) irradiation, for a period of 15 min. The total content of sanguinarine (SA + DHSA) and recoveries were in the range of 4.8-5.8 $mg.ml^{-1}$ and 79-82%, respectively.

A two-dimensional TLC HPTLC coupled with fast atom bombardment mass spectrometry [36] was shown to be especially helpful to survey the amounts of alkaloids with pharmacological activity, including SA, CHE and oxysanguinarine, and to establish the presence of other components in extract of *Sanguinaria canadensis*.

A simple and fast densitometric method using Silica gel 60 F_{254} with chloroform-methanol 60:30 (*v/v*) and methylene chloride-methanol 97:3 (*v/v*) as mobile phases [32] or Sorbfil PTSX-PA-UV silica gel plates using ethylacetate-MeOH-0.1 M NaOH (6:3:2) elution mixture in a pre-saturated chamber [33] were used to quantify the main alkaloids (SA, CHE, chelidonine, coptisine and berberine) in plant organs of *Chelidonium majus*, a well known source of isoquinoline alkaloids of therapeutic value. The application of TLC permitted utilizing the fluorescence of alkaloids without purification and made the detection extremely sensitive. TLC-densitometry is the most convenient analytical technique for routine, fast investigations. The results were compared with those obtained by spectrophotometry (total content of alkaloids as sanguirythrine), GC/MS and HPLC on a C_{18} column using external standards. The results of the chromatographic methods showed good agreement.

Recently, a simple, rapid and reliable TLC method for the separation and identification of sanguinarine obtained from plant tissue cultures was reported.[39]

Chromatographic methods - Gas chromatography/mass spectrometry: The gas chromatographic technique combined with mass selective spectrometric detection (GC/MS) was shown to be a valuable tool and an alternative technique to classical chromatographic techniques and phytochemical procedures permitting the fast separation of alkaloids mixtures and their subsequent identification and quantification.[33, 40] The direct GC/MS analysis of several benzophenanthridine alkaloids (including SA, CHE, DHSA, DHCHE, oxysanguinarine, 11-acetonyldihydrochelerythrine, chelerythridimerine and angoline as the principal constituents) in methanol extract of the bark of *Bocconia arborea* was achieved with a clear distinction between the compounds after fractionation on silica gel.[40]

Crude methanolic extracts of fresh bark of *B. arborea* were dried, extracted with HCl, made alkaline with ammonia and re-extracted with diethyl ether. The vacuum evaporated extracts were re-dissolved in methanol and pre-separated by column chromatography on silica gel with chloroform/methanol (5:1) to yield 7 fractions. The fractions containing alkaloids were analysed by GC/MS using a packed glass capillary column. Using 0.3-0.5 µl injections, eight QBA were identified including SA, DHSA, CHE, DHCHE and their derivatives.[40] Several alkaloids, including SA, CHE and their derivatives, were identified by GC/MS on a CP-Sil 24 CB Low-Bleed/MS quartz capillary column in the homeopathic matrix tincture (HPMT) of *Chelidonium majus* (86% and 62% ethanol, chloroform extracts) evaporated at reduced pressure to dryness and re-dissolved in methanol.[33]

Chromatographic methods - Liquid chromatography: Liquid chromatography combined with UV-VIS spectrophotometric, fluorimetric or mass spectrometric detection is the most effective method for separation, identification and quantification of QBA due to its high separation power, high sensitivity and automations. Normal (Al_2O_3, silica gel *etc*.) and reversed phases (alkylated silica gels C8 – C18), and also polymeric sorbents (with –CN groups etc.) can be applied for separation.

Due to the ionic character of alkaloids in acidic and strongly alkaline mediums, a highly effective ion-pairing chromatography is used. A pH-dependent (acid-base) equilibrium exists between the charged iminium Q^+ and the uncharged (pseudo-base) QOH forms (see above). Ion-pairing reagents (organic amine modifiers, alkylsulfonic acids, i.e. heptane-, hexane- and octanesulfonic acids) in concentrations up to 50 mM are used to avoid the ionic mechanism forming stable ion associates with positively charged iminium ions. The addition of salts (Na^+, NH_4^+, K^+, combined with phosphate, acetate, Br^-, SCN^-, I^-) can be used to decrease retention of cationic species and to eliminate peak tailing in the course of the separation due to a silanol group masking effect. The extended donor-acceptor concept is used to interpret the effectiveness of different salts in masking the active sites of the stationary phase.

RP-HPLC applying alkylated silica gel columns (C8-C18) is currently the most powerful method for QBA separations that allows determination of a variety of groups of alkaloids with different

physico-chemical properties. Highly sensitive direct UV-VIS spectrophotometric and fluorimetric detection are possible since most of the alkaloids contain strong UV-VIS chromophores.

HPLC and CE methods were employed [41] to determine SA in rat hepatocytes, human gingival fibroblasts, feed, porcine faeces, body fluids and tissues. HPLC gradient elution was carried out on a C-18 column under acidic conditions using ion-pairing techniques with the 1-heptanesulfonic acid as ion-pairing agent. A better LOD (3 nM) was obtained for HPLC with fluorimetric detection at $\lambda_{ex/em}$ = 327/577 nm compared to CE/UV-VIS. Both methods are suitable for submicromolar quantities of sanguinarine in biological materials.

A HPLC method utilizing an ethyl silane column with acidic and basic ion-pairing reagents in the mobile phase has been described for the analysis of SA in plant extracts and samples such as saliva and gingival crevicular fluids following a simple acidified methanolic extraction step with a limit of detection of 3 ng in a sample.[56] SA and six common tetrahydroisoquinoline alkaloids (papaverine, noscapine, morphine, codeine, thebaine) and L-tyrosine in *Papaver somniferum* were separated by RP-HPLC in 20 min using a simple water:MeOH linear gradient.[57] Silanol-groups effects commonly associated with the separation of such strongly basic compounds were minimized by the addition of the amine modifier (TEA) to the mobile phase.

A gradient elution RP-HPLC was applied for the determination of tertiary and quaternary alkaloids in *Chelidonium majus* L. extracts [58] on an ODS Hypersil column, using water-ACN-MeOH as an eluent. Replacement of commonly applied organic amine modifiers with KI improved considerably the resolution. Twenty alkaloids were resolved and thirteen tertiary and quaternary alkaloids were identified. Addition of electrolytes (Na^+, NH_4^+, K^+, phosphate, acetate, Br^-, SCN^-, I^-) eliminated peak tailing and decreased retention of cationic species of alkaloids [59] due to a silanol masking effect. The effectiveness of different salts in masking the active sites of the stationary phase is interpreted in terms of extended donor-acceptor concept.

Optimisation of a mobile phase with/without modifiers (ion pair, Na_2HPO_4, TEA) was investigated [50] for preparative HPLC separation of SA and CHE. A larger loading mass and volume were obtained in a shorter time using phosphate buffer/ACN (70/30, v/v) as a mobile phase. Finally, the IP RP-HPLC was not suitable for preparative separation because of its low sample size.

A gradient RP-HPLC separation (0.2% formic acid/water/ACN) on a Nucleosil C 18 column with sensitive fluorimetric detection was performed to investigate the SA biotransformation in cell culture medium, as well as in rat urine and plasma [60] after extraction with an acidified organic solvent. The extraction recovery was about 80% in cell culture medium and in rat urine, but lower in plasma. SA was characterized by MS.

A HPLC method on a Luna C18(2), a new-generation silica-based RP stationary phase, was applied for analysis of isoquinoline alkaloids (SA, protopine, chelidonine, coptisine, and berberine) in acidic methanol extracts of *Chelidonium majus* L [32] further purified by SPE on Supelclean LC-18 cartridges. The same HPLC method with ACN-MeOH-30 mM ammonium formate as a mobile phase was further used to monitor the results obtained by TLC.[52]

Achiral and chiral RP-HPLC methods were applied [61] for separation and quantification of DHSA and its 6-methoxy- and 6-acetonyl- derivatives in methanol extracts of *Hylomecon hylomeconoides* and *H. vernale* (Papaveraceae). Simultaneous separation and determination was possible on ODS column by achiral method using ACN/phosphate buffer as a mobile phase. The same amount of (+) and (-)-enantiomers of DHSA was found using chiral columns.

The detection limits can be improved by some kind of sample preconcentration such as solid phase extraction on C18 cartridges and/or application of the more sensitive fluorimetric detection. The RP-HPLC separation with selective and sensitive fluorimetric detection ($\lambda_{ex/em}$ = 225/410 nm) of benzophenanthridine alkaloids (SA, CHE, chelirubine, chelilutine, sanguilutine, sanguirubine and norsanguinarine) extracted from Papaveraceae sp. cell culture samples [43] allowed their quantification in crude cell extracts and medium samples without any pre-treatment.

An ion-pair HPLC with sodium lauryl sulfate and tartaric acid in ACN/water as eluent was applied for determination of isoquinoline alkaloids in *Argemone ochroleuca* and *A. mexicana* seeds after their extraction into weakly acidified methanol.[62] DHSA and DHCHE (c. 3:2) were found as major alkaloid components (with minor amounts of SA, CHE, protopine, and berberine) in *A. ochroleuca*. DHSA as the major alkaloid with minor amounts of SA and berberine were found in *A. mexicana*, in agreement with earlier studies. DHSA and DHCHE were measured as their oxidized products, SA and CHE after UV irradiation. SA in argemone and other edible oils was separated on a C-18 column with MeOH/ACN/THF/water as a mobile phase and determined at 280 nm by a simple, rapid and reliable RP-HPLC method with the detection limit 5 μg/g.[49]

Chromatographic methods **- Liquid chromatography/mass spectrometry:** Although efficient separations are possible with optical detection, the limiting factor for the method to be widely accepted is a relatively poor limit of detection. The LODs can be improved by application of a more sensitive MS detection. In some cases also ion-exchange sorbents can be used for separation of highly polar alkaloids. The application of mixtures of polar organic solvents (ACN, MeOH *etc*.) in the presence of ammonium salts of formic, trifluoroacetic and other acids allows direct coupling to mass selective detectors. The combination strongly increases the sensitivity of detection (up to fmol range) and allows undisputable identification of alkaloids.

An HPLC/ESI-MS method with detection limit in fmol has been optimized [44, 55] with respect to the individual parameters of both steps - the separation of the HPLC part and ionization conditions of the MS part. The type of sorbent, flow rate of the mobile phase and its composition with respect to the ratio of eluent modifier (electrolyte, ammonium formate) and organic modifier (methanol) are the main parameters responsible for the chromatographic separation.

The effectiveness of HPLC/DAD/ESI–MS separation was tested using the standard solutions of SA, DHSA and BCA (benzo[*c*]acridine) [44] and CHE and DHCHE,[55] respectively, on several reverse phase chromatographic columns C18, C8 and CN. The best results were obtained with a Zorbax SB-CN using a linear gradient separation with a mobile phase consisting of 0.01 M (*v/v*) ammonium formate/methanol. The signal was monitored with the UV-VIS diode array detector and the full spectra were registered in the range of 190–400 nm (SBW 100 nm). The effluent was directly introduced into a quadruple mass spectrometer operated in a positive ESI mode. The *m/z* spectra and data for the selected ion-monitoring (SIM) mode were acquired at *m/z* of molecular ions with LODs in fmol and at *m/z* 200 - 600 for scan mode. The methods were applied to the pharmaco-kinetic studies of the alkaloids in rat.

Nine alkaloids (including SA, CHE and DHCHE) were identified by a HPLC/DAD/ESI–MS) and simultaneously determined [63] by a sensitive HPLC–UV method in 70% methanol extracts of *Zanthoxylum nitidum* on an Agilent C18 analytical column with a gradient elution with ACN/0.1% formate buffer (pH 4.5 with TEA). The contents of alkaloids varied significantly from habitat to habitat and its control is necessary.

Contents of morphine, tetrahydrobenzylisoquinoline, benzo[*c*]phenanthridine, and phtalideisoquinoline alkaloids were determined qualitatively and quantitatively by HPLC and LC-MS analysis [45] in tissues of the Tasmanian *Papaver somniferum* L. and compared with a low-morphine cultivar "Marianne". SA and DHSA were the major alkaloids in the root system. A shifted pattern of alkaloid accumulation and reduced levels of total alkaloid was found in the "Marianne" cultivar. The data suggest a differential alkaloid regulation in each cultivar.

Electromigration methods: In addition to chromatography, electromigration methods have been applied for analyses of alkaloids. Capillary electrophoresis (CE), as a relatively new and still developing method, has already shown a potential for separation, identification and quantification of different groups of alkaloids in biological materials (plant extracts, pharmaceutical preparations, food and feed, body fluids and tissues etc.). CE is suited for the purposes due to high separation efficiency, high separation speed and low sample volumes required.

Usually acidic conditions (phosphate buffer of pH 2.5) in the presence of organic solvents are preferred (MeOH, ACN etc. to increase solubility of alkaloids) as a background electrolyte for CE separations.

Commonly direct or indirect UV-VIS spectrophotometric detection (200-350 nm) are applied since strong absorption of a cationic acid-base form of individual alkaloids appears in UV-VIS region. Fluorimetric detection is seldom used since a highly sensitive laser-induced fluorescence (LIF) detection technique requires analyte derivatization.

The determination of six different isoquinoline alkaloids from *Chelidonium majus* (leaves and roots) using CE was performed in fused silica capillaries with 66 mM phosphate buffer at pH 2.4 containing 10% methanol.[64] SA, CHE, berberine and chelidonine were identified by the on-line UV detection (200-320 nm). Similar conditions (or with 50 mM phosphate-TRIS buffer of pH 2.5/ACN 1:1 v/v as a BGE) were applied for determination of SA and CHE in plant extracts and pharmaceutical preparations [42] and for determination of sanguinarine (50 mM Na phosphate of pH 2.5 with 150 mM SDS) in biological matrices (rat hepatocytes, human gingival fibroblasts, feed, porcine faeces, body fluids and tissues) after pre-concentration by the sweeping effect.[41] Both, CE and HPLC methods are suitable for analysing sub-micromolar quantities of SA and CHE in biological materials and gave comparable results. The HPLC method is usually more sensitive and robust than CE because it uses fluorescence detection and more precise and accurate sample dosing. The CE method was confirmed as a useful complementary technique to HPLC.

Better LODs compared with reported UV-VIS detection could be achieved due to the application of fluorescence detection. The applicability of a highly sensitive laser-induced fluorescence (LIF) detection technique for drug analyses is limited. It usually requires analyte derivatisation, unless the wavelength of native fluorescence of analytes matches the laser ones. A highly sensitive, rapid and simple method of non-aqueous CE with native LIF detection (NA-CE/N-LIF) using a commercially available Ar-laser without troublesome fluorescent derivatisation was applied for the analysis of CHE and SA in *Macleaya cordata* (Willd.) R. Br. and *Chelidonium majus* L.[65]

The capillary electrophoretic/mass spectrometric (CE/MS) separation of four different groups of alkaloids (monoterpenoid indole alkaloids, protoberberines/benzo-phenanthridines, beta-carboline alkaloids, and isoquinolines) from poppy was performed without derivatisation or purification.[66] The total ion current (TIC) and even single-ion mode (SIM) of the $[M+H]^+$ ions modes showed a decreased S/N ratio compared to UV detection and thus better sensitivity. The ESI-MS data of protonated molecules and the Na^+-, and K^+-adducts displayed a typical pattern resulting from cluster formation or doubly charged species. The influence of alkaloid structure on the electrophoretic mobility was also discussed. CE has demonstrated a potential in alkaloids determination and can be regarded as the complementary to the other separation methods such as liquid and gas chromatography. In combination with mass selective detection the sensitivity of CE is sufficiently high for detection of low concentrations of various alkaloids in any type of samples.

BIOTRANSFORMATION OF SANGUINARINE

In vivo studies

The metabolic transformation of sanguinarine occurs in both animal and plant kingdoms with distinct purpose. Whereas in plants it is transformation of sanguinarine as fytoallexine, in animals it is important for its toxicity and elimination as a xenobiotic.

A pioneer work on the metabolic transformation of sanguinarine in animals was published in the late 50s´. The author found that sanguinarine is converted to a green-fluorescent compound in isolated rat liver.[67] In an ongoing work, another research group injected lactating rabbits subcutaneously with sanguinarine. They described the transmission of sanguinarine to the milk of lactating rabbits (also cats, rats and monkeys), where it was bound to albumin. They also reported the presence of a green-fluorescing metabolic product in the urine of injected rabbits. Based on comparative UV spectroscopy they concluded that this putative metabolite might be 3,4-benzacridine.[31] However, one drawback of this study was the isolation procedure that used drastic hydrolysis by hydrochloric acid thus causing artifacts. Biometabolic elimination and organ retention of sanguinarine was again studied after more than thirty years.[68] Rats and guinea pigs were administered a single oral dose of a mixture of sanguinarine and dihydrosanguinarine. The composition of this mixture was not defined since it was obtained by precipitation of the alkaloids from argemone oil by hydrochloric acid. Thin layer chromatography analyses showed that after 72 h of treatment sanguinarine and dihydrosanguinarine disappeared and a new green fluorescence spot appeared in the chromatogram of collected urine and feces samples. The retardation factor of this spot was between those of sanguinarine and dihydrosanguinarine and the authors interpreted this spot as 3,4-benzacridine.[68] However, this interpretation was not supported by any direct analytical/structural evidence. The authors also showed that sanguinarine and dihydrosanguinarine are excreted to the bile of rats and guinea pigs. However, no green-fluorescence metabolite was detected in the bile.[9, 68] Our recent findings suggest that the formation of dihydrosanguinarine might be the first step in sanguinarine detoxification in the rat and its subsequent elimination in phase II reactions. Benzo[c]acridine, was found neither in urine nor in plasma and liver [44]. A follow up study further confirmed this hypothesis.[69] This is in conformity with the finding that sanguinarine reductase is a key enzyme in sanguinarine detoxication in plants.[70]

In vivo assessment of sanguiritrin (mixture of sanguinarine and chelerythrine) safety to animals was performed recently. In 90 days feeding experiment in swine, daily oral dose of alkaloids up to 5 mg per 1 kg animal body weight proved to be safe.[71] The follow up study investigated subchronic safety of SANGUIRITRIN in rats. Animals were fed a diet containing 120 ppm sanguiritrin for 109 days. It was evidenced that 2% of QBA were absorbed through the GIT while 98% were excreted in the feces. No adverse effects were observed on rat organism.[72]

Interesting is a possible interaction between sanguinarine and the cytochrome P450 system. A single intraperitoneal administration of argemone oil in rats caused the destruction of hepatic cytochrome P450 content and consequent inhibition of monooxygenases activity.[73] Sanguinarine cytotoxicity may be associated with aryl hydrocarbon receptor (AhR) signaling pathways, tentatively with CYP1A. Pretreatment of mice with 3-methylcholanthrene (3-MC), an inducer of CYP1A enzymes, mitigated the sanguinarine toxic effects.[74]

In vitro studies

There are multiple indications in the literature, that sanguinarine biological activity, cytotoxicity and/or metabolism could be associated with the AhR/CYP1A system. It was found that sanguinarine inhibits catalytic activity of CYP1A enzymes [75] but has no effect on dioxin-inducible expression of AhR-dependent genes.[76-79] On the other hand, dioxin-mediated activation of AhR led to the attenuation of sanguinarine cytotoxicty in HepG2 cells [75] and rat hepatocytes.[80] This finding correlates with the effects observed in vivo.[74]

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