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Review

Polyhydroxy alkaloids: chromatographic analysis

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Abstract

Polyhydroxy alkaloids are a burgeoning category of natural products that encompass several structural types and generally exhibit potent activity as inhibitors of glycosidases. As presently defined the group consists of monocyclic or bicyclic alkaloids of the pyrrolidine, piperidine, pyrrolizidine, indolizidine and tropane classes, bearing two or more hydroxyl groups. These structural features render the compounds highly water soluble and frequently quite insoluble in non-hydroxylic solvents, so that their isolation and analysis by chromatographic means are consequently difficult. This problem is further confounded by the lack of a chromophore which would permit their detection by UV absorption. This review presents chromatographic techniques that have been successfully applied to the problem of isolating, purifying, detecting and analyzing polyhydroxy alkaloids.
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1. Introduction

The discovery of polyhydroxy alkaloids with glycosidase inhibitory properties has taken place primarily within the past two decades. The indolizidine alkaloids swainsonine and castanospermine [1,2] which possess potent and specific inhibitory activities towards α-mannosidase [3] and α- and β-glucosidase [4], respectively, stimulated the search for new members of the class that could be considered to be nitrogen-containing analogs of simple sugars and might therefore also have similar glycosidase-inhibitory properties. The importance of such properties resides in the influence of inhibitors upon the fundamental cellular function of glycoprotein processing, physical and biochemical consequences therefrom, and their resultant potential application as antiviral, anticancer and antibiotic agents, in addition to studies and treatment of other disease states [5]. Consequently, about 50 naturally occurring members of the group, including glycoside derivatives, have been isolated and identified from plants and microorganisms and their occurrence and biological properties have been the subject of a recent extensive review [6]. In addition, many more synthetic analogs have been prepared which enlarges the class to the order of 400 individual compounds [7]. However, the chromatographic properties of the latter differ little, if at all, from the natural alkaloids and this review therefore does not discriminate between polyhydroxy alkaloids from natural or synthetic sources.

Although these alkaloids cannot be represented as a single class they possess sufficient commonality in many of their features, including two or more hydroxyl groups and a heterocyclic nitrogen atom, for them to be integrated into structural groups based upon five- and six-membered rings, which may also be fused into bicyclic ring systems. The simple monocyclic systems, together with the more complex bicyclic rings, comprise five different sub-classes, namely: pyrrolidines, piperidines, pyrrolizidines, and indolizidines (Fig. 1), and nor-tropanes (Fig. 2).

Alkaloids of the five-membered ring pyrrolidine class are represented by 2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine (DMDP) (**1**) [8], while 1-deoxynojirimycin (DNJ) (**2**) [9] is typical of the six-membered ring piperidine group. Polyhydroxy

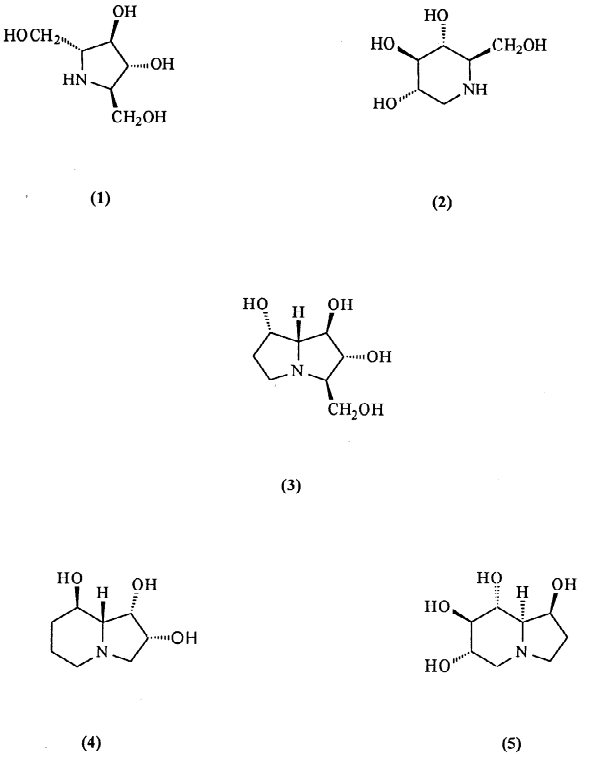

Fig. 1. Representative structures of polyhydroxy alkaloids of the pyrrolidine, piperidine, pyrrolizidine and indolizidine classes: 2*R*,5*R*-dihydroxymethyl-3*R*,4*R*-dihydroxypyrrolidine (DMDP) (**1**); 1-deoxynojirimycin (DNJ) (**2**); australine (**3**); swainsonine (**4**); castanospermine (**5**).

pyrrolizidine alkaloids, exemplified by australine (**3**) [10], may be considered in a formal structural sense to be the result of fusion of two pyrrolidine ring systems, with the common nitrogen atom at the bridgehead. Similarly, the indolizidine alkaloids may be visualized as a pyrrolidine ring fused to a piperidine ring moiety, yielding a bicyclic five/six ring system, with swainsonine (**4**) [1,11–13] and castanospermine (**5**) [2] being particularly important members of this family.

An alternative fusion of a five-membered pyrrolidine ring with a six-membered piperidine ring at positions α- to the nitrogen atom of each monocyclic system gives rise to a nor-tropane ring system (Fig. 2). Polyhydroxy alkaloids of this type have been named calystegines after the source of the first member to be isolated, the bindweed *Calystegia sepium* [14,15]. Three subclasses have been defined,

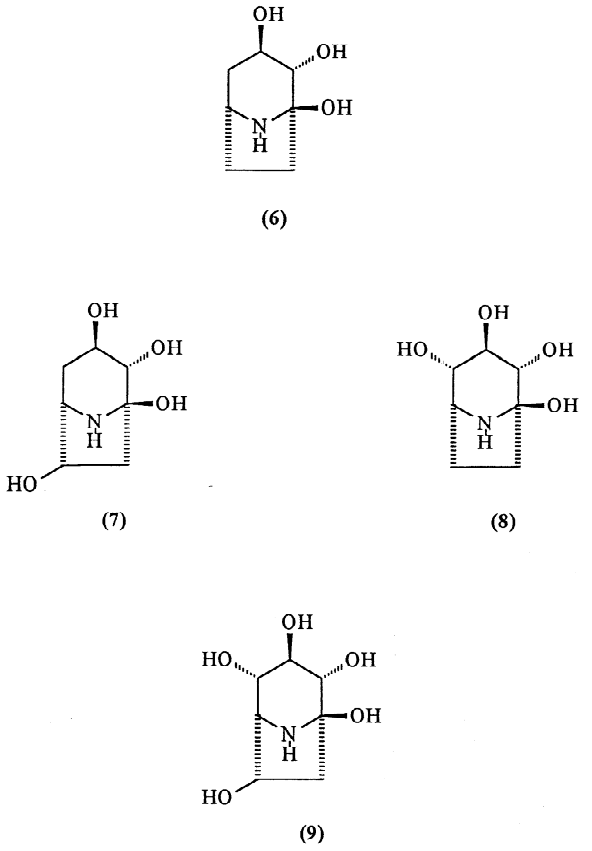

Fig. 2. Representative structures of polyhydroxy alkaloids of the nor-tropane classes: calystegine A_3 (**6**); calystegine B_1 (**7**); calystegine B_2 (**8**); calystegine C_1 (**9**).

calystegines A, B, and C, bearing three, four, and five hydroxyl groups, respectively. Calystegine A_3 (**6**) is representative of the first class, calystegines B_1 (**7**) and B_2 (**8**) of the second, and calystegine C_1 (**9**) is prototypical of the most highly hydroxylated group. A consistent feature of all calystegines is the presence of an α-OH group at the bridgehead juncture of the bicyclic ring system adjacent to the nitrogen atom, which can be regarded as an amino-hemiketal moiety. Although these alkaloids are a relatively new addition to the polyhydroxy glycosidase inhibitors the group now consists of more individual alkaloids than any of the other classes. The recent discovery of *N*-methylcalystegine B_2 and *N*-methylcalystegine C_1 [16] has broadened the group beyond the nor-tropane class sensu stricto to encompass the tropane alkaloids.

Other combinations of the pyrrolidine and piperidine rings can be envisioned. An obvious construct is the fusion of two piperidine moieties to generate a quinolizidine system. Although polyhydroxyquinolizidine alkaloids have not yet been discovered in nature, ring-expanded analogs of castanospermine have been synthesized which show significant glycosidase inhibitory activity [17,18].

The polyhydroxy alkaloid glycosidase inhibitors encompassed by these structural types have been extensively reviewed either individually or collectively with respect to their chemistry, mode of action and biological properties [5,6,19–22].

Structural elucidation of polyhydroxyalkaloids is dependent on the application of the most sophisticated physical techniques. Mass spectrometry provides fundamental information with regard to the molecular formula, ring size and general substitution pattern but may provide little information with regard to relative distribution of hydroxyl groups around the ring system; it also has no value for isomers which are epimeric with respect to various hydroxy groups or at the bridgehead in bicyclic systems. This information can generally be obtained by nuclear magnetic resonance spectroscopy, particularly two-dimensional and nuclear Overhauser effect methods, which are most suitable for elucidating interconnectivities and relative stereochemistry. Determination of absolute stereochemistry necessitates the use of either X-ray crystallography or circular dichroism (CD). The value of all of these techniques is conditional upon the alkaloid under investigation being obtained in as pure a form as possible and to achieve this chromatographic methods must be applied to isolation, purification and analysis [23,24].

2. Extraction and chromatographic isolation

Polyhydroxy alkaloids have commonly been extracted from ground plant material using methanol or ethanol, frequently admixed with 25–50% water, and on occasion with water alone. Dilute acid has also been added [25] and while it is doubtful that this increases the efficiency in comparison to a continuous Soxhlet extraction, the presence of acid may suppress the decomposition of certain pyrrolidines, which has been observed to occur at high pH [23].

Swainsonine (**4**) was initially extracted with ethyl acetate [1] and acetone [11] but in general the use of alcohol–water solvent mixtures is most appropriate because of the high solubility of this class of alkaloids in polar solvents and their quite typical lack of solubility in non-polar solvents. A few of the less highly hydroxylated alkaloids, primarily those bearing only two hydroxyl groups, have some degree of solubility in solvents such as chloroform [26], which may permit them to be separated from their more highly hydroxylated congeners. Nevertheless, they still retain significant hydrophilicity and their differential solubility between chloroform and water is far too low for this solvent to be used to extract them from basified aqueous solution, as is the usual procedure for purification of alkaloids.

A consequence of the use of aqueous and/or alcoholic solvents is the co-extraction of many other polar constituents such as sugars and amino acids, together with the desired polyhydroxy alkaloids. This necessitates a significant purification effort to remove extraneous compounds, which is best achieved through ion-exchange chromatography. A number of different resins have been used, in both anionic and cationic forms, either Dowex 50 or Amberlite CG120 in their NH_4^+ or H^+ ion forms being most commonly employed. The isolation of castanospermine (**5**) [2] and a dihydroxypyrrolidine alkaloid [27] from seeds of *Castanospermum australe* exemplifies this procedure. The crude extract is suspended in water, acidified with hydrochloric acid to ca. pH 4 and insoluble material removed by centrifugation or filtration. It may be advantageous to remove fats and waxes, which can encapsulate the alkaloids and decrease their water-solubility, by extraction with a solvent such as ether or methylene chloride [28]. However, this is not often done and it may be more convenient to defat the plant material prior to extraction with the polar solvent.

The acidified aqueous solution is applied to the column and the unretained neutral or acidic substances are eluted with water. The alkaloids which are bound to the resin, accompanied by any other non-alkaloidal basic compounds, commonly aspartic acid and other basic amino acids, are then displaced with dilute ammonium hydroxide. In a less commonly used variation of this procedure, the alkaloids are retained on Dowex 50 in its pyridinium or hydrogen ion form, and non-basic substances are displaced with 1–2 *M* pyridine followed by elution of the alkaloids with dilute ammonium hydroxide. This method appears to be particularly suitable for pyrrolidine alkaloids such as DMDP (**1**) [8,27,29].

Alkaloids derived from pipecolic acid may require a somewhat different ion-exchange purification method from that of other polyhydroxy alkaloids because the carboxyl functionality renders them much less basic. Thus, dihydroxypipecolic acids have been obtained from leaves of *Calliandra* species by preliminary purification on a CG-120 resin in its hydrogen ion form and elution with dilute ammonium hydroxide, followed by further separation on a Dowex 50 (H^+) resin with elution by 1–2 N hydrochloric acid [30,31]. In contrast, the carboxylic acid analog of australine (**3**), 7a-*epi*alexaflorine, a constituent of the leaves of the rain-forest tree *Alexa grandiflora*, was isolated by conventional cation exchange chromatography [32].

Following ion-exchange purification, those alkaloids that exist as solids may be sufficiently pure to be crystallized after elution from the column, although this is generally not possible when the alkaloidal mixture is fairly complex. However, castanospermine (**5**) can be crystallized easily from extracts of *Castanospermum australe* seeds because it is the predominant alkaloid and most of its co-occurring epimers and isomers are oils [33–35]. Others, such as di- and tri-hydroxypyrrolidines [27,29], and the piperidine alkaloids fagomine [36] and dihydroxypipecolic acid [31] have been crystallized as their hydrochloride salts, which are particularly suitable for X-ray structure elucidation. Yields of the alkaloids are often quite low, in most cases not exceeding 0.1%. However, a trihydroxypyrrolidine has been isolated from ripe fruits of *Angylocalyx boutiqueanus* in 0.16% yield [27], and yields as high as 0.47% have been obtained for castanospermine (**5**) from undried seeds of *Castanospermum australe* [37].

Chromatographic isolation has been extended further by the use of polyhydroxy alkaloids themselves as affinity chromatography ligands for the specific binding and purification of glycosidases involved in glycoprotein trimming reactions. The pyrrolidine alkaloid, 1-deoxynojirimycin (**2**), and its D-manno- and D-galacto-analogs, have been converted to *N*-

carboxypentyl derivatives and covalently bound to Sepharose 4B resin for use in purifying the enzyme to homogeneity [38,39].

A combination extraction–purification method has recently been applied to the measurement of swainsonine in locoweed populations of the genus *Oxytropis*. Liquid–liquid extraction and partitioning of small (100 mg) samples between chloroform and 2% acetic acid was followed by rapid concentration of the alkaloid by solid-phase extraction of the acidic portion onto a short Dowex 50W-X8 column from which the alkaloid was eluted with 1 *M* ammonium hydroxide [40]. This eluate was then analyzed directly by atmospheric pressure chemical ionization tandem mass spectrometry (see Section 4.4).

3. Preparative chromatographic separation

3.1. Column chromatography

As an extension of the ion-exchange purification process, certain alkaloids have been separated by collection of fractions from the column. For example, the indolizidine alkaloid castanospermine (**5**) elutes from the column before the pyrrolizidine alkaloid australine (**3**) and its epimers, despite the fact that they all have the same number of hydroxyl groups [41,42]. However, the selection of fraction sizes is inherently arbitrary and the lack of UV absorbance requires that less convenient methods must be used to detect the compounds and establish their purity. In some cases separation has been achieved by employing up to five different resin types in sequence, as in the isolation of a trihydroxypipecolic acid from *Baphia racemosa* [43], but this procedure seems unnecessarily complex. Nevertheless, it may be required when the extract is found to contain a significant number of closely related alkaloids, as is the case with the calystegines in *Lycium chinense* [16] and the 11 pyrrolidine and piperidine alkaloids which have been isolated from bulbs of bluebells, *Hyacinthus orientalis* [44].

The initial purification of swainsonine was achieved by chromatography on the weak cation-exchange resin CM Sepharose CL-6B by a linear gradient of sodium chloride (0–1 *M*) in sodium acetate (10 m*M*, pH 5), the elution being followed by enzyme inhibition [1]. Active fractions were pooled and lyophilized and the swainsonine extracted with ammoniacal chloroform and crystallized. Calystegine fractions A and B (i.e., the trihydroxylated and tetrahydroxylated groups) were separated by passage through a GF 05 gel filtration column using the volatile buffer (pH 7) trimethylammonium carbonate [15] but column chromatography on polar packing materials other than ion-exchange resins has been applied only rarely because the basicity and polyhydroxy substitution pattern of the alkaloids results in strong adsorption on silica gel and other such materials. This requires the use of very polar solvent mixtures to elute the desired compounds. However, swainsonine (**4**), isolated from the microorganism *Metarhizium anisopliae*, was separated by elution from silica gel with *n*-butanol–ethanol–chloroform–28% ammonium hydroxide (4:4:4:1) [13]. Less polar packing materials have also been used and several polyhydroxy pyrrolidine alkaloids separated in this way. Thus, 1-deoxymannojirimycin, the mannose analog of 1-deoxynojirimycin (**2**) has been obtained by elution from an alumina column with 90% ethanol [25] and fagomine was first obtained by using silica gel–Celite (2:1) with elution by 8.8% ammonium hydroxide–isopropanol (1:4) in water [36]. Calystegines have also been purified after ion-exchange chromatography by separation on Sephadex LH-20 in the reversed-phase mode using *n*-butanol–acetic acid–water–methanol (10:10:90:15) [45]. Additionally, a trihydroxypyrrolidine has been separated from an extract of fronds of the fern *Arachnioides standishii* by elution with *n*-butanol–pyridine–water (1:1:1) from a cellulose column [29], a procedure which is essentially a scale-up of preparative paper chromatography.

3.2. Paper chromatography

Preparative paper chromatographic (PC) separation was employed to advantage in the earliest separation of polyhydroxypipecolic acid alkaloids but has not been applied to other classes. Ascending PC on Whatman 3MM paper with 80% phenol–water (w/v) in the presence of ammonia vapor [30,31] or with *n*-butanol–formic acid–water (15:3:2) [46] have been the methods of choice. A solvent system consisting of *t*-amyl alcohol–

lutidine–water (89:89:57) has also been used. The bands can be detected with ninhydrin and the individual alkaloids extracted from the paper with large volumes of water. Paper chromatography has now been superceded by thin-layer chromatography although it may offer some advantages in the case of a few extremely polar alkaloids.

3.3. Thin-layer chromatography

Unless the volume of alkaloid extract is exceptionally large, preparative thin-layer chromatography (TLC) separation techniques are generally faster and more convenient than column chromatography, involving less expenditure for materials and solvents. Preparative TLC has therefore been the separation method of choice for the isolation of individual polyhydroxy alkaloids from mixtures, with silica gel being the most suitable stationary phase in both conventional preparative TLC and centrifugal radial TLC applications. Whereas the former offers advantages of convenience and minimal expense, major problems arise in detecting the alkaloid bands on the plate and separation from the stationary phase. The polar solvents necessary to elute the alkaloids often result in dissolution of silica from the substrate and consequent contamination of the purified sample with material which may be very difficult to remove completely. Centrifugal radial TLC [47] combines high separation speed with flexibility in fraction sizes collected, so that major components can be pooled, while alkaloids with very similar R_F values may be separated by collection of small fractions. Individual fractions, or representative samples, can be tested for purity by several methods, including analytical TLC or gas chromatography and their potency of enzyme inhibition may also be determined. The technique permits large (gram) quantities of crude mixture to be rapidly separated, and with excellent reproducibility once a suitable separation procedure has been developed, while contamination with dissolved silica is not a problem. The primary disadvantages are the initial expense of the equipment and the larger quantities of solvent required than for conventional TLC, although the amounts are considerably less than for column chromatography.

The solvents used for preparative TLC have generally been combinations of chloroform, methanol and ammonium hydroxide. The dihydroxyindolizidine alkaloids lentiginosine and 2-*epi*lentiginosine, which are minor constituents of the spotted locoweed (*Astragalus lentiginosus*), were separated from each other and from the major component swainsonine (**4**) by TLC on 0.25-mm plates using chloroform–methanol–ammonium hydroxide–water (70:26:2:2) [26]. Swainsonine was also isolated from extracts of the mold *Rhizoctonia leguminicola* on silica gel G plates, using acetone–chloroform–50% aqueous diethylamine (60:20:40) [48].

The minor alkaloids 6-*epi*castanospermine, 6,7-di*epi*castanospermine, 7-deoxy-6-*epi*castanospermine and australine (**3**) in the mother liquors remaining after crystallization of castanospermine (**5**), the major alkaloid of the Moreton Bay chestnut (*Castanospermum australe*), have been separated by centrifugal radial chromatography on 2-mm silica gel plates, with sequential development using chloroform–methanol–ammonium hydroxide–water (82.5:15.5:1:1 and 70:26:2:2), followed by 2% ammonium hydroxide in ethanol [10,33–35].

TLC fractionation of polyhydroxy alkaloid mixtures on silica gel after peracetylation has been used to isolate swainsonine (**4**) [12] and 1-*epi*australine [28]. The acetate derivatives are much less polar and therefore more mobile than their parent alkaloids and relatively non-polar solvents (2–4% methanol in chloroform) can be used to develop the TLC plates, while they can be extracted from the matrix without the use of hydroxylated solvents likely to solubilize some of the silica. However, the fractions obtained in this way cannot be screened for glycosidase inhibition unless they are first hydrolyzed with methanolic potassium carbonate. Also, rearrangement or degradation of a particularly labile alkaloid may potentially occur during the acetylation–deacetylation process, even though the conditions are relatively mild. Nevertheless, the technique is particularly useful for isolation of specific alkaloids once their identity has been established unequivocally as a naturally occurring component of the plant extract.

4. Chromatographic analysis and detection

There is no doubt that the polyhydroxy alkaloid

class remained undiscovered until relatively recently due to the difficulty of detecting them with conventional alkaloid-specific reagents. As a consequence, few methods have yet been developed for their analysis and detection. However, these compounds have generated much interest not only because of their glycosidase inhibitory properties but also because of their potential as therapeutic agents for treatment of various disease states [5,6,21]. Analytical methods are therefore needed to detect and measure the alkaloids in plants or fungi and to determine their distribution in biological fluids such as blood and serum. Some specific analytical methods have already been developed and these will undoubtedly be improved in sensitivity and specificity, while novel techniques will increasingly be applied to the problem.

4.1. High-voltage electrophoresis

High-voltage paper electrophoresis (HVE) has been applied most successfully to the hydroxypipecolic acid alkaloids, using a pH 1.9 buffer at 60 V/cm [31]. Two-dimensional chromatography consisting of uni-directional development by HVE followed by development in the other direction by conventional paper chromatography (see Section 3.2) permitted the resolution of all 8 *cis*/*trans* isomers of mono- and di-hydroxypipecolic acids. These compounds are more readily detected than the non-carboxylic alkaloids since they give a green color with ninhydrin, fluoresce brick-red under UV, and give a positive reaction with isatin. Analysis of these alkaloids can also be performed on a commercial amino acid analyser adapted for fluorimetric detection [49].

A more general method for examining extracts containing the alkaloids by HVE was subsequently developed [50]. Samples and appropriate standards, such as known alkaloids and amino acids, are subjected to a current of 5 kV for 30 min using acetic acid–formic acid–water (148:33:1819) (pH 1.9) or acetic acid–pyridine–water (138:15:2347) (pH 3.6) buffers. The papers may be sprayed with ninhydrin, which generates a yellow to yellow–brown color with the alkaloids, or with nitroprusside reagent, which yields blue colors with many of the alkaloids. Most of the alkaloids show mobilities similar to arginine. A screening for polyhydroxy alkaloids by HVE, with ninhydrin detection, established the presence of 2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine (DMDP) (**1**) in species from several genera of the family Araceae [51].

Calystegines were first detected in root exudates from the bindweeds, *Calystegia sepium* and *Convolvulus arvensis*, as well as from *Atropa belladonna*, by paper electrophoresis at 3 kV in a pH 1.9 buffer consisting of formic acid–acetic acid–water (3:6:91) [14]. The analysis was achieved in 15 min and the alkaloids were visualized by dipping the paper in a solution of silver nitrate in aqueous acetone, followed by aqueous ethanolic sodium hydroxide, and fixed with photographic paper fixer.

4.2. Thin-layer chromatography

Analytical TLC has proved to be particularly applicable to the polyhydroxy alkaloids for purity determination and detection in plant extracts [52] and for pharmacokinetic studies [53]. Chromatographic conditions are essentially the same as for preparative TLC (see Section 3.3). The primary drawback is that this class of compounds is remarkably insensitive to reagents commonly used for alkaloid visualization, such as Dragendorff's or iodoplatinate, giving a consistent reaction only when very high concentrations of alkaloid extract are applied to the TLC plate. Many react with ninhydrin to give somewhat nondescript yellowish or buff colors which are not particularly diagnostic. With this reagent the speed of color development is highly variable among individual alkaloids, although the pyrrolidines tend to react more rapidly than piperidines. The polyhydroxypipecolic acid alkaloids give more distinctive color reactions, similar to those observed for HVE analysis (see Section 4.1).

A specific TLC detection reagent has been developed for the polyhydroxyindolizidines such as swainsonine (**4**) and castanospermine (**5**) [52] which are structurally capable of generating a pyrrole moiety by dehydration, either directly or via rearrangement. This utilizes a dual reagent spray system consisting of 10% acetic anhydride in benzene, followed by Ehrlich's reagent (*p*-dimethylaminobenzaldehyde in ethanol containing boron trifluoride etherate), after which an intense

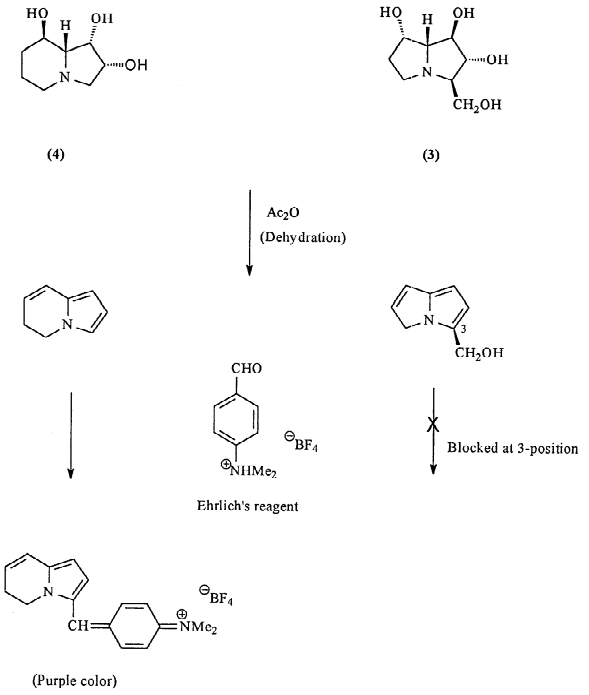

Fig. 3. Hypothetical reaction of Ehrlich's thin layer spray reagent with swainsonine (**4**) and australine (**3**).

purple color is generated on heating the plate. The method is highly sensitive, with a minimum detection level for most alkaloids of about 0.5 μg. The Ehrlich's reagent presumably condenses at the position α- to the nitrogen atom in the pyrrole ring (Fig. 3). *N*-Oxide derivatives also react since one degree of unsaturation is introduced by a Polonovski rearrangement analogous to that of pyrrolizidine alkaloids [54]. Presumably pyrrolidine alkaloids having a free α- position would react similarly, although this has not been established. Australine (**3**) and its epimers give a much less distinctive pink coloration, possibly because dehydration occurs primarily in the most highly substituted of the two pyrrolidine rings which has the α- position blocked by a hydroxymethyl substituent (Fig. 3). Condensation with Ehrlich's reagent would therefore require rearrangement of the double bonds into the least substituted pyrrolidine ring of the overall pyrrolizidine ring system, a process unlikely to occur with any degree of facility once a stable pyrrolic moiety has already been formed.

TLC on silica gel plates with the solvent system *n*-propanol–acetic acid–water (4:1:1) has been applied successfully to several different classes of polyhydroxy alkaloids and the chlorine–*o*-tolidine spray reagent, which was originally developed for amino acids and peptides [55], has been used for detection [44]. An extensive comparison of calystegine detection on silica gel, polyamide, and kieselguhr as stationary phases, developed with various solvent systems, and visualized with ninhydrin, sodium prusside–sodium carbonate–acetaldehyde, Ehrlich's reagent, or silver nitrate–ethanolic sodium hydroxide, has been performed [56]. Silica gel TLC was eventually selected as a preliminary screening tool to separate calystegine groups A, B, and C (Fig. 2) from one another, with R_F values of 0.49–0.52, 0.41–0.51, and 0.33, respectively. The solvent system consisted of methanol–chloroform–ammonium hydroxide–water (46:50:1:3) [57] with the plates being developed twice and the alkaloids detected with silver nitrate–sodium hydroxide dip reagents [14].

4.3. Gas chromatography and gas chromatography–mass spectrometry

The structural relationship of polyhydroxy alkaloids to sugars has pointed the way to gas chromatography (GC) as a technique for analysis either of extracts after ion-exchange chromatography or of separated components. As with the sugars, the underivatized alkaloids are too polar for GC in their native form but trimethylsilyl (TMS) derivatization of the hydroxyl groups provides them with the necessary volatility and stability for GC analysis.

Trimethylsilylation of polyhydroxy alkaloids belonging to several different structural classes was first achieved with trimethylchlorosilane–hexamethyldisilazane–pyridine (1:3:9) at 50 °C for 15 min, although a trihydroxypipecolic acid had to be heated at 60 °C for 1 h [58]. It is probable that other polyhydroxypipecolic acid derived alkaloids would also require these more forcing conditions due to facile derivatization being suppressed through hydrogen bonding of the carboxylic acid with either the adjacent imino or hydroxyl groups; alternatively, the carboxylate group may reduce the propensity for derivatization of α-hydroxy groups through steric

hindrance. The derivatized alkaloids were separated on a 5-ft×4-mm I.D. glass column packed with Chromosorb W HP coated with either 3% OV-1 or OV-17, with the column temperature programmed from 135–175 to 125–200 °C, respectively, and detection by flame ionization. All of the pure alkaloids analyzed gave single peaks and although the extent of trimethylsilylation was not determined it is likely to be complete under the conditions reported. Retention times ranged from 2.0–17.9 min on OV-1 and 1.3–12.9 min on OV-17 and there was a close correlation of retention time with degree of hydroxylation [58]. Thus, a simple dihydroxypyrrolidine eluted fastest and the tetrahydroxyindolizidine, castanospermine (**5**), had the longest retention time on both column packings. Separation of all of the alkaloids was not complete on either column, but the retention times differed from one column to the other, so that the alkaloids could be resolved by dual analysis. Packed column GC with 3% OV-1 on Chromosorb W (2-m×2-mm I.D.) has been used to analyze various plant parts of five *Swainsona* species for the presence of swainsonine and swainsonine *N*-oxide. Castanospermine was added as an internal standard and the sample was derivatized with hexamethyldisilazane–trifluoroacetic acid–pyridine (9:1:10). In a standard mixture, the *N*-oxide eluted first, followed by the free base and finally castanospermine, but no swainsonine *N*-oxide was detected in the plant samples [59].

Superior resolution to packed-column GC analysis, with complete separation of all alkaloids, including epimers [10,26,33–35,60], has been achieved using a 30-m×0.32-mm I.D. SE-30 fused-silica capillary with on-column injection. Detection was by flame ionization with the column programmed from 105 to 300 °C. Derivatization reagents which give involatile by-products cannot be used however since the injection needle or capillary may become blocked and the most suitable reagent was found to be *N*-methyl-*N*-trimethylsilyltrifluoracetamide (MSTFA) in pyridine. This reagent yields only highly volatile by-products, but the sample must be maintained at 60 °C for 1 h to ensure that all of the alkaloids give single peaks. Mass spectrometric analysis of the TMS derivative of swainsonine (**4**) showed that complete derivatization is achieved under these conditions, as well as with the silylation reagents bis(trimethylsilyl)trifluoroacetamide (BSTFA) and hexamethyldisilazane–trimethylchlorosilane. However, although castanospermine (**5**) was completely derivatized with MSTFA, it gave three major peaks on treatment with BSTFA even after prolonged heating [61]. Additional experiments with swainsonine and castanospermine have shown that the detector response is linear over a wide range of concentrations, with a minimum detection level of about 150 pg. This method has therefore been used to determine the swainsonine content of locoweed plant samples, without the necessity of adding an internal standard [61].

Quantitation of calystegines in transformed root cultures of *Atropa belladonna* and *Hyoscyamus muticus* has been done, with simultaneous detection by a flame-ionization detector (FID) using octadecane as an internal standard, and by a phosphorus nitrogen selective detector (PND) using deoxynojirimycin (**2**) as an internal standard [56,62]. The TMS derivatives were prepared by treatment with hexamethyldisilazane–trichlorosilane (10:1) in pyridine at 50 °C for 15 min and the product chromatographed on a 30-m×0.25-mm I.D. DB5 column with a 1:20 split ratio injection. With an injection volume of 1 μl, the detection limit for a calystegine was ca. 60 pmol for FID and ca. 10 pmol for PND [56], demonstrating the enhanced sensitivity of the latter detector which should also reduce the signal complexity by its lack of response to non-nitrogenous constituents. The identity of the TMS derivatives as those in which the nitrogen atom was underivatized was established by chemical ionization mass spectrometry using ammonia as a reagent gas.

The problem of partial derivatization is the major drawback to the use of TMS derivatives for GC analysis and is illustrated by recent experiments with the nor-tropane group. Treatment of the trihydroxy alkaloid, calystegine A_3 (**6**), and the tetrahydroxy alkaloids, calystegines B_1 (**7**) and B_2 (**8**) (Fig. 2), with MSTFA in pyridine at 60 °C for 12 h initially gave pertrimethylsilylated derivatives in which all hydroxyl groups and the secondary amino group were derivatized, although shorter reaction times led to partial derivatization [63]. However, in subsequent experiments, designed to detect the calystegines and other polyhydroxy alkaloids in extracts from *Ipomoea* species [64,65] the amino group remained

underivatized even under forcing conditions of high temperature and extremely long reaction times. It seems probable that samples of calystegines may have to be stringently dried in order for the amino group to react but this is not necessary because complete derivatization of all hydroxy groups occurs under the moderate temperatures and reaction times used for all the other alkaloid classes, and these derivatives are sufficiently volatile for analysis. Treatment of all of the known classes of polyhydroxy alkaloids with MSTFA at 60 °C for 1 h is therefore universally applicable for derivatization, and as with the packed-column GC analysis, the capillary column retention times correlate closely with the degree of hydroxylation (R.J. Molyneux, unpublished data) (Fig. 4). As a result it is generally possible to estimate the number of hydroxyl groups in a novel alkaloid from its retention time.

In combination with mass spectrometry (GC–MS) the GC technique shows great potential for analysis of polyhydroxy alkaloids in plant samples and even animal tissue samples. As an example, swainsonine (**4**) and calystegines B_2 (**8**) and C_1 (**9**) (Fig. 2) have been shown to co-occur in *Ipomoea calobra* and *I. polpha* from Australia [64], *I. carnea* from Mozambique [65] (Fig. 5), and *I. asarifolia* from Brazil (R.J. Molyneux, unpublished data). All of these species have been reported to cause poisoning of livestock in these countries and the identification of specific toxins, in association with pathological evidence, has confirmed their mode of toxicity as induced lysosomal poisoning diseases. The distribution of calystegines in the plant family Convolvulaceae and their significance as taxonomic markers has been studied by GC–MS analysis of 65 species belonging to 22 genera. Seven individual calystegines were detected by chromatography on a 30-m×0.25-mm DB-1 column and between one and five of these alkaloids were found in 30 species belonging to 15 genera [57]. A more limited study of the Solanaceae was done in a similar manner [66] and the distribution of calystegines in eight species of this family using analogous methods has recently been reported [67].

A considerable amount of structural information may be gained regarding the components of an alkaloidal mixture by GC–MS analysis. The fragmentation pattern of the TMS derivatives of swainsonine and castanospermine is analogous to that of the underivatized alkaloids, showing cleavage of the pyrrolidine ring at the bonds β- to the nitrogen atom [1,61]. Thus, not only can the total number of hydroxyl groups be determined but also their distribution between the five- and six-membered ring portions of the molecule as a whole. Alkaloids that possess a pendant hydroxymethyl group, such as DMDP (**1**) and australine (**3**), exhibit an intense fragment ion corresponding to loss of m/z 103, due to cleavage of the $-CH_2OTMS$ moiety from the molecular ion [10,68]. Another characteristic fragmentation is the appearance of an ion at m/z 217, corresponding to the presence of three adjacent hydroxyl groups, a common structural feature of many of the calystegines, and fragmentation patterns have been hypothesized for both the per-TMS and the *O*-TMS calystegine derivatives [56,63,67].

Recently, an extensive study has been made of the conditions required to derivatize the hydroxyl groups, the amino group, or both, for all eight known mono- and di-hydroxypipecolic acids [69]. All of the reagents employed gave varying amounts of -*O*-TMS and -*N*-TMS derivatives but derivatization of the hydroxyl groups alone could be induced by freeze-drying the samples and then exposing them to atmospheric moisture prior to treatment with trimethylchlorosilane–hexamethyldisilazane–pyridine (1:3:9). Unfortunately, the use of MSTFA for the specific formation of -*O*-TMS derivatives was not investigated. The derivatives were well separated on a 25-m×0.2-mm I.D. BPX5 capillary column using a somewhat complex temperature gradient. Although the GC–MS analysis was conducted in the EI mode, a protonated molecular ion $[M+H]^+$ was observed for all of the standards, and the expected fragment due to loss of m/z 117, corresponding to –COOTMS, was always present. The technique was applied to a taxonomic evaluation of the genus *Inga* by analysis of herbarium leaf samples from 11 different species [69] and also to establish a relationship between the aroid tribes Nephthytideae and Aglaonemateae, supported by the accumulation of significant amounts of the polyhydroxy-pyrrolidine and -piperidine alkaloids, 2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine (DMDP) and α-homonojirimycin and its epimers [70], respectively. This application illustrates the value of the GC–MS

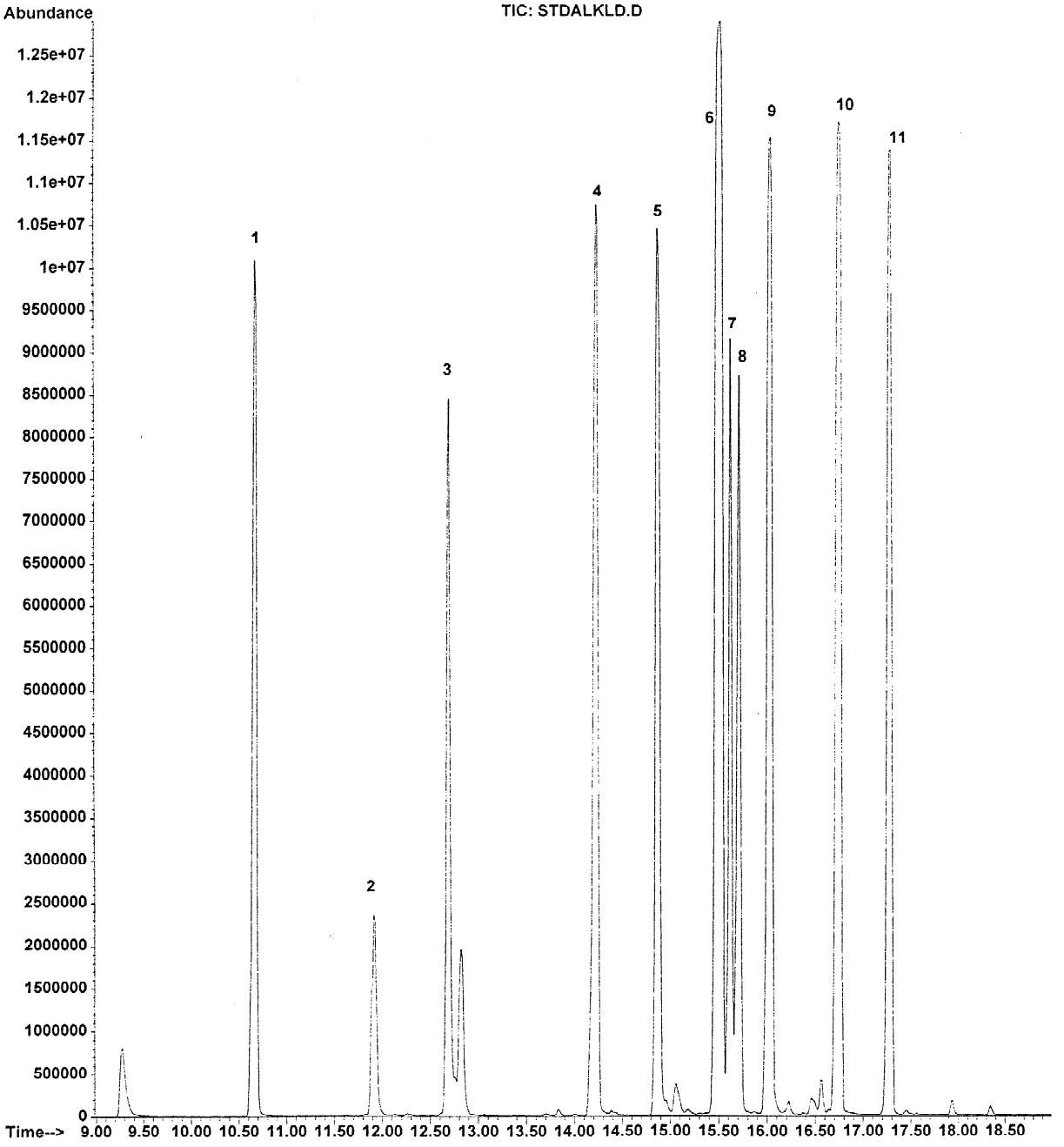

Fig. 4. GC–MS separation of a mixture of selected polyhydroxy alkaloids as TMS derivatives. (1) Aspartic acid; (2) platynecine; (3) fagomine; (4) swainsonine (**4**); (5) 1-deoxynojirimycin (**2**); (6) australine (**3**); (7) 1-*epi*australine; (8) 3-*epi*australine; (9) 6-*epi*-castanospermine; (10) castanospermine (**5**); (11) castanospermine *N*-oxide.

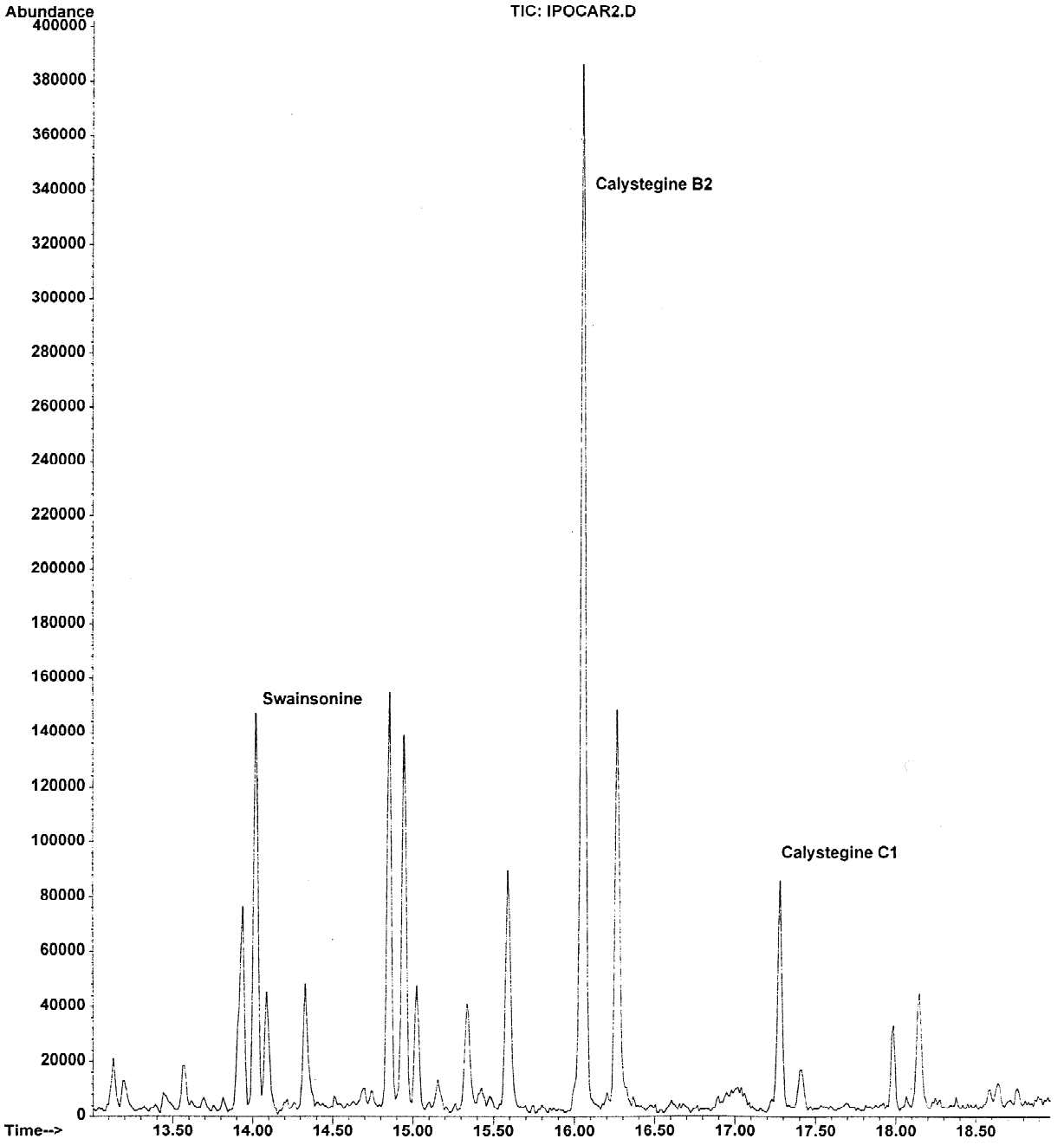

Fig. 5. GC–MS identification of polyhydroxy alkaloids swainsonine (**4**), calystegine B_2 (**8**), and calystegine C_1 (**9**) in *Ipomoea calobra*.

technique for chemotaxonomic examination of plant samples because the high sensitivity and small sample size required yields a considerable amount of information from material which may be botanically valuable or not readily available [71]. For example, it is conceivable that it could be used to identify residual plant material in the gastrointestinal tract of poisoned animals.

Further potential of the technique is illustrated by the use of GC–MS to elucidate the metabolism of synthetic 6-*O*-butanoylcastanospermine (MDL 28,574: BUCAST) in cells infected with herpes simplex type II virus [72]. The results established that castanospermine (**5**) was the active intracellular metabolite but that the precursor derivative was more effective against the virus, presumably because the lipophilicity of the butanoyl moiety increased uptake and enhanced transport into the infected cells. An approach that did not use the TMS derivative was employed in clinical studies to measure the pharmacokinetics of swainsonine in the serum of cancer patients receiving the alkaloid in a phase I clinical trial. Prior to GC–FID and GC–MS analysis on a 15-m×0.53-mm I.D. DB225 capillary column the pure alkaloid or serum extracts were peracetylated by treatment with acetic anhydride and 4-dimethylaminopyridine [73]. By the use of methyl α-D-mannopyranoside and methyl β-D-galactopyranoside as internal standards, serum concentrations of swainsonine were shown to increase rapidly during the first 8 h after commencement of intravenous infusion and ultimately attained levels ca. 100–400 times greater than the 50% inhibitory concentration of Golgi α-mannosidase II. Undoubtedly, the application of GC techniques will become of increasing importance in clinical studies of this type as the therapeutic potential of these alkaloids is exploited further.

4.4. High-performance liquid chromatography and liquid chromatography–mass spectrometry

As a consequence of their lack of a suitable chromophore for spectroscopic detection conventional high-performance liquid chromatographic (HPLC) analysis has rarely been applied to the polyhydroxy alkaloids. The detection problem could be surmounted by pre- or post-column derivatization of the hydroxyl groups with an appropriate chromophore or fluorophore, but this adds to the complexity of the analysis. A more serious problem is that the extreme hydrophilicity and generally low solubility in non-hydroxylic organic solvents impedes the development of suitable solvent system–column packing combinations.

The problem of detection of the alkaloids during HPLC analysis has been overcome to some extent by use of a pulsed amperometric detector [74]. Deoxynojirimycin (**2**), deoxymannojirimycin, swainsonine (**4**) and castanospermine (**5**), were separated with a Dionex CS3 cation exchange column under isocratic conditions using 10 m*M* hydrochloric acid at a flow-rate of 1 ml/min as the mobile phase. These four alkaloids were then detected on a gold working electrode with a linear response over a concentration range of 10 ng/ml to 20 μg/ml after post-column addition of 300 m*M* sodium hydroxide. The alkaloids showed base-line separation on the ion-exchange column, with a run-time of less than 20 min but the lower detection limits ranged from 10 to 50 ng/ml and the sensitivity of the method is not comparable with GC methods.

The use of mass spectrometry as a liquid chromatography detector has obvious advantages and thermospray LC–MS experiments have shown promising results with the alkaloid mixture from seeds of *Castanospermum australe* [37]. Preliminary fractionation of the crude extract was performed on a 250×22.5-mm I.D. preparative Phenomenex IB-SIL 5 NH_2 column using an acetonitrile–water gradient as the mobile phase. Fractions were then chromatographed at 1 ml/min on a 250×4.6-mm I.D. column containing a cation-exchange packing (Partisil 10 SCX) in acetonitrile–water (5:95) containing 0.015 N ammonium formate. Thermospray ionization was generated by the NH_4^+ ions present in the mobile phase. Under these conditions of soft ionization only protonated molecular ions were obtained, no fragment ions being observed, and fagomine, australine (**3**), castanospermine (**5**), and 6-*epi*castanospermine were separated and detected. The detection level for castanospermine, using single-ion monitoring, was ca. 500 pg.

An unidentified peak was also observed with a mass-to-charge ratio (m/z) of 174, corresponding to a trihydroxyindolizidine, which could be due to 7-deoxy-6-*epi*castanospermine [35]. In addition, one of the fractions from the preparative column was found to give a major peak at m/z 190 which eluted slightly after australine (**3**). Further analysis by thermospray LC–MS, using an amino column (Carbo, 250×4.6-mm I.D.) eluted with acetonitrile–water (80:20) at 1 ml/min and ionization with

filament-on mode, resolved this peak into three individual peaks. The major component showed a fragment ion at m/z 158, corresponding to loss of a hydroxymethyl group, and it seems probable that these three peaks correspond to australine epimers, of which 1-*epi*- and 3-*epi*australine have been isolated [28,42,75].

These results indicated that further development of the LC–MS technique, particularly electrospray ionization (EI) and atmospheric pressure chemical ionization (APCI), would prove useful for the analysis and identification of novel polyhydroxy alkaloids from plants and microorganisms. A comparison of GC–MS, LC–sequential MS (MS^n), and direct infusion APCI–MS, applied to a partially purified extract of polyhydroxy alkaloids from *Hyacinthiodes non-scripta*, indicated that the latter technique was best for rapid screening of extracts [76]. However, LC–MS was better for separation and identification of isomeric alkaloids. In contrast, GC–MS suffered from the necessity of having to prepare sufficiently volatile derivatives, which may lead to the formation of several products if the derivatization reaction is not carefully controlled, and the inability to resolve and obtain useful mass spectra from alkaloid glycosides with molecular masses exceeding the range of the MS detector after derivatization. Negative ion MS–MS, using an APCI source has been demonstrated to be suitable for the identification of mono- and di-hydroxypipecolic acid isomers and their respective epimers, whereas analyses conducted in the positive ion mode were less successful at discriminating between the epimers [77].

Direct MS analysis has been utilized for the detection of polyhydroxy alkaloids in methanolic plant extracts partially purified by adsorption onto a Dowex 50 (H^+) ion-exchange resin and elution with aqueous ammonium hydroxide [78]. Most of the compounds which could potentially interfere with the MS analysis are thus removed from the matrix, since only basic constituents of the extract are retained. Samples were introduced into a quadrupole ion-trap mass spectrometer with an APCI source operated in the positive ion mode by infusion with a syringe pump into a flow of 50% aqueous methanol regulated at 0.5 ml/min with an HPLC pump. First-order MS gave $[M+H]^+$ ions and subsequent MS^2 and MS^3 product ion fragmentations were characterized by sequential losses of hydroxyl groups as water. The nature of such patterns does not permit discrimination between isomers or epimers but the ability to screen relatively crude extracts provides fundamental information with respect to the general classes of polyhydroxy alkaloids likely to be present and their degree of hydroxylation. Extracts of *Omphalea diandra*, *Syzygium oleosum*, *Hyacinthiodes non-scripta*, and *Castanospermum australe*, four plant species either known or suspected to contain polyhydroxy alkaloids, were examined in order to evaluate the utility of the method. In addition to known alkaloids, hexose diglycosides of DMDP (**1**) and α-homonojirimycin or an isomer were detected for the first time in *O. diandra*, and a pentose diglycoside of the pentahydroxy pyrrolizidine alkaloid, casuarine, in *S. oleosum*. The previously reported homoDMDP-7-*O*-apioside [79] was detected in *H. non-scripta* and there was evidence for the presence of a hexose glycoside of castanospermine (**5**), or one of its isomers, in *C. australe*. In contrast to the parent alkaloids, which show successive losses of water in the MS^2 product ion spectra, the glycosides exhibited losses of either 162 or 132 a.m.u, corresponding to hexose or pentose residues, respectively. The resulting MS^3 product ion spectra then showed losses of 18 a.m.u. identical to those observed for the MS^2 spectra of the non-glycosylated alkaloids. Although the technique does not provide complete structural elucidation of the alkaloids present in an extract, these examples illustrate the advantages of the LC–MS^n technique in providing fundamental information with respect to both alkaloid aglycones and glycosides which may be present, in a single experiment.

The LC–MS approach has recently been evaluated in some detail by comparison of APCI and electrospray (ES) sources with 12 polyhydroxy alkaloids encompassing all of the known structural classes [80]. Chromatography was accomplished on a 150× 4.6-mm I.D., 7-μm Adsorbosphere XL Carbohydrate AX column, with an acetonitrile–water gradient. APCI in the positive mode was a more effective ion source than ES, ionizing all of the alkaloids tested but the deprotonated $[M-H]^-$ molecular ions produced in the negative mode APCI proved to be more diagnostic in distinguishing between isomeric alkaloids. Detection levels in the positive mode APCI

were in the low picogram range using extracted ion chromatograms for the $[M+H]^+$ ion, whereas ES only permitted detection at the nanogram level. These results indicate that preliminary HPLC separation, in combination with both positive- and negative-mode APCI detection, should permit detection and primary structural characterization of polyhydroxy alkaloids in a complex mixture.

A practical application of the LC–MS–MS technique has been demonstrated with the detection and analysis of swainsonine (**4**) in various populations of locoweeds in the genus *Oxytropis* [40]. After liquid–liquid extraction and preliminary purification by solid-phase extraction (see Section 2), HPLC was performed on a 100×2-mm I.D. Betasil C_{18} reversed-phase column with an isocratic mixture of 5% methanol in 20 m*M* aqueous ammonium acetate; swainsonine eluted at a retention time of 1.4 min. Detection and quantitation was achieved by APCI in the positive ion mode with sequential tandem mass spectrometry (MS^2). The first-order mass spectrum of swainsonine showed an intense protonated molecular ion at m/z 174 and an MS^2 product ion spectrum at m/z 156, corresponding to loss of a single hydroxyl group as water. Attempts to quantitate the swainsonine by direct injection and tandem mass spectrometry gave erratic results but prior HPLC separation gave consistent measurements with an increase in analysis time of 5 min or less. The detection limit of swainsonine was 0.019 mg/ml, corresponding to 0.001% swainsonine by weight in dry plant material. Analysis of plant material from 16 sites in the Western United States showed that only five populations of *Oxytropis lambertii* var. *bigelovii* in the extreme southwestern areas contained swainsonine at levels greater than 0.001%. The *O. lambertii* varieties *articulata* and *lambertii* contained no swainsonine at any of the sites sampled. Analysis of *O. sericea* from three locations historically associated with livestock poisoning, collected over a 3-year period, showed great variation in swainsonine content with location and between years. Individual plants also exhibited high variability, with some plants in a population containing no measurable swainsonine. These findings suggest that swainsonine concentrations may be associated with infection of the plant by an endophyte [40]. The results demonstrate the potential utility of the LC–MS method for studying and predicting the potential for further episodes of livestock poisoning.

4.5. High-performance liquid chromatography and glycosidase inhibition

A particular advantage of HPLC methods that do not employ destructive detection techniques is that post-detection eluate fractions can be collected and assayed for glycosidase inhibitory activity after adjustment to pH 4.8 with a phosphate buffer. This is a valuable asset, since the importance of this group of alkaloids resides to a great extent in the biological effects that accrue from this property. The measurement of glycosidase inhibitory activity is essentially a colorimetric method which depends upon the generation of *p*-nitrophenol (UV absorbance at 410 nm) or 4-methylumbelliferone (fluorescence emission at 449 nm on irradiation at 365 nm), respectively, from a *p*-nitrophenyl or 4-methylumbelliferyl glycosidic substrate by the appropriate enzyme [3,4,33,81]. In the presence of a glycosidase inhibitor the release of the chromophore is suppressed and the existence of an alkaloid in any particular fraction could therefore be detected by the absence of color relative to the solvent blank. For most purposes the fractions would probably need to be screened against several enzymes, each requiring its own substrate, and the best application of the method would therefore be to collect fractions and conduct the assay in a multi-well microtiter plate. A number of glycosidases are commercially available, including yeast α-glucosidase, almond emulsin β-glucosidase, coffee bean α-galactosidase, bovine liver β-galactosidase, jack bean α-mannosidase, bovine epididymus α-L-fucosidase, *Aspergillus niger* β-xylosidase, bovine liver β-glucuronidase and bovine kidney β-*N*-acetylhexosaminidase. Alternatively, enzymes may be obtained from animal, plant or microbial sources, particularly when there is reason to believe that an alkaloid may possess novel or specific inhibitory activity [82,83].

Although the combination of this technique with HPLC has not yet been attempted, its value as an analytical technique for glycosidase inhibitors has been demonstrated by its use in first isolating swainsonine (**4**) by monitoring fractionation of extracts [1] and to measure yields of the same alkaloid

from the fungus *Metarhizium anisopliae* under various culture conditions [84]. The method has also been used to determine swainsonine levels in the serum of cattle and sheep ingesting the locoweeds *Astragalus lentiginosus* and *Oxytropis sericea* [85], as well as in a comparative study of the lesions produced in rats by feeding *Astragalus mollissimus*, swainsonine (**4**) and castanospermine (**5**) [86]. Such assays can also be performed after purification of the plant extract by ion-exchange chromatography (see Section 3.1) to remove interfering substances such as polyphenolics which are themselves capable of glycosidase inhibition.

5. Conclusions

At present the most useful general analytical technique for polyhydroxy alkaloids is capillary gas chromatography, which provides such excellent resolution that even epimers can be separated, and in combination with mass spectrometry (GC–MS) provides a great deal of structural information. However, liquid chromatography will undoubtedly become the greatest area of growth as bench-top LC–MS instruments become easier to operate, more reasonable in price, and thus more widely distributed. The ability to analyze the polyhydroxy alkaloids and their glycosides without derivatization will confer a particular advantage upon this technique.

In comparison with most other classes of alkaloids, the polyhydroxy glycosidase inhibitors are a relatively recently discovered group. This is undoubtedly a consequence of their water-solubility and cryptic nature. Nevertheless, they have been found to occur in a wide diversity of plant families, as well as in certain microorganisms, and at the present time there does not appear to be a taxonomic pattern governing their distribution. This is exemplified by swainsonine (**4**) which was first discovered in the genera *Swainsona*, *Astragalus* and *Oxytropis* of the plant family Leguminosae, subsequently identified as metabolites of the fungi *Rhizoctonia leguminicola* and *Metarhizium anisopliae*, and more recently detected in *Ipomoea* species in co-occurrence with calystegines B_2 (**8**) and C_1 (**9**). This implies that these compounds probably serve an important function related to their glycosidase-inhibitory properties in both plants and fungi, and many as yet unknown sources and novel structural types therefore wait to be revealed. Such discoveries will be entirely dependent upon the application of the most sensitive chromatographic techniques for detection, purification and analysis. In view of their significance as natural toxins, biochemical probes, and potential drugs, the present limited choice of techniques can be expected to grow considerably in the future.

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