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RADIOIMMUNOASSAY DETERMINATION OF SCOPOLAMINE YIELDS IN TISSUE CULTURES OF *DATURA*

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Brett J. Savary

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ABSTRACT

The purpose of this study was to establish a radioimmunoassay (RIA) and determine its applicability for quantifying scopolamine yields in plant cell cultures of Datura. Scopolamine binding antisera were obtained using the immunogen described by Weiler et al. (1981). A RIA was established based on the commercially prepared (N-methyl[^{3}H])-scopolamine methylchloride as labelled antigen. The linear range of the scopolamine standard curve extended from 0.15 to 1.5 ng in the assay mixture. The standard curve was routinely reproduced, and the precision of triplicates, measured as the coefficient of variation for B/B_{0}, was ca. 2.3%. The specificities of two selected antisera were characterized for the cross-reactivities of metabolically related and structurally similar tropane alkaloids.

Callus tissue cultures were established from leaf explants of five species of Datura. Simple ethanol extracts were prepared and assayed with the RIA. Antigen activity was detected in all culture lines. Cultures derived from plants of D. stramonium consistantly averaged the highest yields of antigen activity by the RIA when grown on B5 medium (Gamborg, 1970) supplemented with 1 mg/L 2,4-dichlorophenoxyacetic acid. Variations in activity yields were observed between species and between lines established from different individual plants of a species.

An extract of D. stramonium callus tissue was separated by reverse phase HPLC and the collected fractions were assayed with the RIA to determine if the antigenic activity measured was specifically due to scopolamine. Five antigenic activity peaks were recognized.

Correspondence of elution times of the activity peaks with both internal and external spikes of alkaloid standards gave tentative identification for scopolamine, 6-hydroxyhyoscyamine, hyoscyamine, and littorine as components of the extract. Hyoscyamine was determined to be the major antigenic component and was present in the callus extract at a 25-fold quantity greater than scopolamine. Thus it was determined the RIA cannot be used to selectively quantitate scopolamine in simple extracts of callus tissue of D. stramonium because a low cross-reacting compound was accumulated in an excess quantity sufficient to overwhelm the specificity of the antiserum. These results indicate the application of any "specific" RIA to tissue culture extracts needs a verification to determine if low cross-reacting compounds are accumulated in excess of the compound of interest at levels sufficient to invalidate the specificity of the antiserum.

The KLH-10 RIA described here can potentially be applied to simple extracts of callus tissue if a solid-phase extraction purification step (e.g., by a Sep Pak) is introduced to the preparation of extracts before RIA analysis. Purification by a solid-phase extraction cartridge can be optimized for the selective removel of hyoscyamine from D. stramonium callus extracts before RIA analysis. The HSA-12 RIA can be applied to unpurified extracts for the determination of scopolamine-type tropane alkaloids as a group. Alternatively, if an RIA is required that must specifically quantitate scopolamine in unpurified tissue culture extracts, then an new immunogen must be prepared with scopolamine conjugated to carrier protein through the tropic acid moiety, and antisera must be produced and screened to obtain an antiserum with a higher specificity for scopolamine.

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CHAPTER I

INTRODUCTION

Secondary Products in Plants

Many higher plants produce specific chemicals that are useful to humans. Those phytochemicals referred to as secondary products have diverse uses which include flavors, fragrances, dyes, pesticides, and medicinals (Dougall, 1979; Balandrin et al., 1985). The biosynthesis of secondary products is ubiquitous through the plant kingdom, but the production of specific types of these compounds is typically restricted to a few families or genera of plants. Secondary products are compounds of low molecular weight (<2000) and are derived from primary metabolites through specific enzyme mediated biosynthetic pathways. Secondary products comprise a vast spectrum of compounds and are classified according to their biosynthetic derivation and chemical structure. The different classes include alkaloids, flavonoids, quinones, tannins, steroids and terpenoids.

Secondary products are generally synthesized and accumulated in differentiated tissues at specific developmental stages during the lifecyle of the plant. This developmental pattern is defined and regulated by the genetic material (Krikorian and Steward, 1969). Many secondary products appear to be non-essential for the survival of the cell but rather have an ecological function in the survival of the whole plant. These include herbivore feeding deterents, phytoalexin responses to pathogen invasion, pollinator attractants, and allelochemics in inter-plant competition. In contrast, primary

metabolites are common cellular constituents of all plant species and whose biosynthesis is essential for growth, maintenance, and reproduction of the plant cell.

Plant secondary products are important commodities for several industries and have values that can range from the hundreds (e.g. quinine) to the thousands of dollars per kilogram (e.g. shikonin) (Curtin, 1983; Balandrin et al., 1985). The economic importance of secondary products is exemplified by those used as medicinals and which must be extracted from plants. These constituted 25% of all prescriptions dispensed in community pharmacies in the United States during 1973 and this represented an estimated annual cost to consumers of about $1.6 billion (Phillipson, 1979).

Most desirable secondary products can be chemically synthesized, but this method is usually not economical and so they must be obtained from natural sources. Political, economic, environmental, and ecological factors can, however, limit an adequate supply of natural plant products (Staba, 1969; Zenk, 1978; Dougall, 1979). As an alternative source to direct chemical synthesis or natural collection, the use of plant cell cultures has been considered for the commercial production of natural products (Zenk, 1978; Dougall, 1979; Curtin, 1983).

Secondary Products in Plant Cell Cultures

The ability of plant cells to synthesize and accumulate specific secondary products in unorganized callus and suspension cultures has been well established and extensively discussed (see Staba, 1985; also

Zenk, 1978; Dougall, 1979 and 1985; Yamada and Hashimoto, 1984). Mitsui Petrochemical Industries of Japan has successfully applied this to the commercial production of shikonin (Fujita and Tabata, 1987), and there are currently six other plant cell culture systems which are either operational or at pilot plant stage (Fowler, 1987). However, the number of systems with potential application for the commercial production of secondary metabolites is limited. Systems with demonstrated productivities that approach or exceed the yields from plants represent only a fraction of the number of compounds investigated and whose production by tissue cultures is desired (Dougall, 1985). Those systems exhibiting high yields were obtained by improvement of culture conditions and by selection of high-producing cultures or cell lines (Dougall, 1985). Despite the limited number of successes, the understanding of the behavior of plant cells grown in vitro has improved, and this knowledge provides an empirical approach for achieving higher yields.

Approaches for Increasing Culture Yields

Zenk and coworkers have outlined a strategy for isolating cell culture strains which yield substantial amounts of secondary products (Zenk et al., 1977; Deus and Zenk, 1982): 1) Development of a suitable analytical method. 2) selection of plants with high contents of the desired compound; 3) development of a production medium; and 4) selection of high-yielding variant cell lines.

A suitable analytical method is necessary to efficiently screen cultures for the isolation of high-yielding cell lines. Visual

selection with the naked eye can be used when the desired compound is colored. This was used for establishing high yielding cultures of the red pigment shikonin (Mizukami et al., 1978) and for fluorescent compounds with the fluorescent microscope (Deus and Zenk, 1982). For compounds such as alkaloids which usually are neither colored nor fluorescent, the radioimmunoassay and related techniques have been used (Zenk et al., 1977). Absorption microspectrophotometry has been applied to the analysis of rosmarinic acid in single cells (Chaprin and Ellis, 1984). Recently, the cell sorter (flow cytometer) has allowed the combined isolation and determination of berberine content of individual protoplasts (Fujita and Tabata, 1987).

Individual plants within a species exhibit a wide range of specific yields, and cultures initiated from high-yielding individual plants tend to give higher yields (Zenk et al., 1977). Different species of plants which produce the same specific compound exhibit different yields in culture, i.e. there is no correlation between the amounts produced in the plants of different species and the amounts produced in the cultures derived from the plants (Schulte et al., 1984). Therefore many different high yielding plants from different species which produce the target compound should be used to initiate cultures which could then be screened for high yielding lines (Dougall, 1985).

Medium composition and cultural conditions can be optimized to increase the productivities of plant cell cultures (Zenk et. al., 1977; Dougall, 1979). The production medium optimized for anthraquinone yields by cell suspenion cultures of Morinda citrifolia resulted in

yields that exceeded those of differentiated root tissue by a factor of 10 on a dry weight basis (Zenk et al., 1975). These results were obtained by optimizing the quality and quantity of the growth regulators used and nutritional factors such as carbohydrates, nitrogen, phosphate, and vitamines contained in the medium.

An optimized production medium often does not support adequate growth of the plant cells and a two-stage culture is used (Zenk et. al., 1977). In the first stage the cells are grown in a growth medium developed for increasing the biomass of the culture. In the second stage the cells are maintained in a production medium optimized for secondary product biosynthesis. The two-stage culture is necessary for those systems where cell growth and biosynthetic activity is incompatible and product formation occurs only after cell growth declines or ceases. Other systems exhibit a production-growth pattern where product formation proceeds almost in parallel with cell growth and a single medium is optimized for both cellular processes (Tabata, 1977).

Selection of Variant Cellular Phenotypes

The ability of a plant cell grown in vitro to accumulate the characteristic secondary metabolite may be regarded as a specific cellular phenotype because this accumulation by a cell requires specific biochemical and cytological specializations that arise from differential expression of the genetic material (Krikorian and Steward, 1969; Luckner and Nover, 1977). Variation in accumulating phenotypes occurs in cultured plant cells and this variation affects the

productivity of cultures (Zenk, 1978; Dougall, 1985). This variation may be due to different cell types derived from the explant and by heritable changes in cellular phenotypes that regularly arise during culture (Meins, 1983).

The variation in accumulating phenotypes that exists in a culture can be exploited to improve yields of secondary products by selection and establishment of variant cell lines with yields that match or even exceed those of differentiated plant tissue (Zenk et al., 1977; Dougall, 1979). High yielding variant cell lines can even be obtained from cultures that produce the characteristic secondary metabolite at very low levels (Tabata and Hiraoka, 1976).

Sources of Heritable Variation

Variation in culture productivity attributable to heterogeneity of phenotypes isolated in the original tissue explant can be maintained even after many subcultures. This can occur through stabilization of different phenotypes through cell-cell interactions and by the relative growth rates of the different phenotypes (Meins, 1983). This source of variation is indicated by isolating single cells and showing significant differences in the yields of cultures derived from them. This approach was used by Tabata and Hiraoka (1976) to demonstrate differences in alkaloid production by callus cultures of Nicotiana rustica. In this study free cells were isolated from callus tissue which had been in culture for only a few passages. Cultures derived from these cells were shown to exhibit wide differences in growth and nicotine production. Variarion attributable to different cell types

originating from the explant was also indicated by Constable et al. (1981) who determined qualitative variations in the alkaloid spectra of 76 clones derived from protoplasts isolated from a single leaf.

Plant cells grown in vitro regularly undergo heritable changes in cellular phenotypes (Meins, 1983). A phenotypic change is determined to be heritable when it is stable through cellular divisions and persists in the absence of the inducing event, and these changes in heritable cellular phenotypes occur through both genetic and epigenetic phenomena (Meins, 1983). Genetic changes occur through a permanent physical alteration of the genetic material and effect the phenotype of a cell by altering the genotype. Epigenetic changes occur through reversible alterations in the pattern(s) of gene expression and are not the result of permanent changes in genetic material. The mechanisms involved in epigenetic changes are not understood.

Heritable changes attributable to interconversions of accumulation phenotypes that occur during culture are demonstrated by the isolation of subclones from a clone of known productivity and showing these subclones exhibit significant differences in the level of accumulation (Meins, 1983). Ogino et. al. (1978) examined subclones from a callus culture of N. tabacum derived from a clone selected for high nicotine content, and substantial variation in nicotine content was observed. Dougall et al. (1980) examined the properties of anthocyanin accumulation by both high and low accumulating clones from a wild-carrot (Daucus carota) suspension culture. The populations of subclones isolated from both types of clones were shown to have become heterogenous after maintenance by serial passage. The

demonstration that low accumulating clones gave rise to higher accumulating subclones, in addition to the lower yielding subclones derived from the high accumulating clone, indicated the ability to accumulate anthocyanin in reversible.

The ability of wild-carrot cells to specifically synthesize anthocyanin and the upper limit in anthocyanin accumulation (Dougall et al., 1980) are reflective of the genetic capacity directing anthocyanin biosynthesis. However, the apparent high rates of variation in the ability of clones to accumulate anthocyanin, the reversibility of the ability to accumulate, and the observation that the rate of loss of ability to accumulate anthocyanin is different in different culture media (Dougall et al., 1980) are indicative of epigenetic regulation involved in the differential expression of the genetic material.

Investigations concerning of the biochemical basis of heritable variation in secondary product accumulating phenotypes can help to better understand the regulatory mechanisms involved in the expression of such cellular phenotypes. Control of these mechanisms could then allow a broader biotechnological application of plant cell culture for the production of useful secondary metabolites.

Description and Rationale of Thesis Project

The establishment and application of an RIA to the determination of scopolamine yields in tissue cultures of Datura is described in this thesis. This is the first description of the application of a scopolamine RIA to tissue cultures. The purpose of the project was to verify the applicability of the RIA to simple extracts of Datura cells

grown in culture and thereby determine if Datura can be used as an in vitro model system for investigations concerning intracellular variation in scopolamine production and for other studies concerning scopolamine metabolism.

The establishment of this RIA involved the production of scopolamine-binding antisera, the adaptation of the RIA procedure (described by Weiler and coworkers) to meet our applications and facilities, and the characterization of the specificities of two antisera selected for use in the RIA. The RIA that was established was then applied to callus cultures of five species of Datura to determine if they were producing antigenic activity. This thesis also describes the examination of the antigenic composition of a D. stramonium culture line by the separation of an extract using high performance liquid chromatography (HPLC) and RIA analysis of the collected fractions to determine the applicability of the RIA to simple extracts.

Considerations for the selection of Datura as the system for study include the following: 1) The culturability of Datura has been well established in the literature. 2) Datura is a primary source for the commercially important tropane alkaloids scopolamine and hyoscyamine (Griffin, 1976); These are two of the most commonly prescribed pure compounds (pharmaceuticals) derived from higher plants (Phillipson, 1979), and they have significant values to warrant consideration of their production by plant cell cultures (Zenk, 1978). 3) The biosynthesis and metabolism of the tropane alkaloids has been investigated (Evans, 1979; Leete, 1979), and this background provides a

framework for *in vitro* studies of scopolamine biosynthesis. 4) A competitive binding RIA for the determination of scopolamine content in plant tissues of *D. innoxia* has been described (Weiler et al., 1981).

Pharmacology and Biosynthesis

Scopolamine and hyoscyamine are classified as anticholinergics and parasympatholytics. Scopolamine is commonly used for treating motion sickness, as a sedative, and as a preanesthetic. Hyoscyamine is used as a mydriatic, suppressent of gastric and bronchial secretions, and for treatment of poisoning by organophosphate pesticides and *Amanita muscaria* ingestion (Cordell, 1981).

Biosynthesis of hyoscyamine occurs in the roots of plants by the esterification of tropine with tropic acid (Leete, 1979). The pyrrolidine ring of tropine is derived from ornithine, and tropic acid is derived from phenylalanine with intramolecular rearrangement of the carboxyl side group (Leete, 1979). Scopolamine is formed from hyoscyamine via 6-hydroxyhyoscyamine (Hashimoto and Yamada, 1986) with much of this transformation occuring in the aerial parts of the plant after translocation (Plank and Wagner, 1986).

Radioimmunoassay of Scopolamine

The radioimmunoassay is based on the competition between the unknown amount of unlabelled antigen and a fixed quantity of labelled antigen (tracer) for binding to a limited amount of antibodies. The free fraction and the (antibody) bound fraction in an assay mixture are separated and the radioactivity in either phase is measured. Because the amount of tracer bound by the antiserum is a function of the

quantity of unlabelled antigen present in an assay mixture, the quantity of antigen in a sample added to the assay mixture can then be determined by comparison with a standard curve (Weiler, 1977).

The radioimmunoassay has been demonstrated to be a very efficient, specific and highly sensitive analytical technique for quantifying secondary metabolites (Weiler, 1977) and would thereby be better suited than conventional chromatographic techniques for investigating variation in yields of scopolamine by tissue cultures.

The various colorimetric and chromatographic methods that have been used to quantitate scopolamine extracted from plant tissues or pharmaceutical preparations have sensitivites in the lower microgram range (Weiler et. al., 1981). But more recently TLC-densitometry (Deuz et al., 1985), GLC-FID and GC/MS (Yamada and Hashimoto, 1982), and HPLC-UV_{204} (Plank and Wagner, 1986) methods have been described with sensitivities in the 20-200 ug range. Weiler et al. (1981) reported a RIA for the quantitative measurement of scoplamine with a sensitivity of 0.2 ng; An approximate 100-1000 fold increase in sensitivity is thus realized by the RIA. A high sensitivity is necessary for determination of alkaloid yields of unorganized cultures of Datura because alkaloid biosynthesis occurs at levels much lower than the plant (Hiraoka and Tabata, 1974). A higher sensitivity also allows the analysis of smaller quantities of tissue.

The extreme specificity that an antibody can potentially exert for an antigen provides the basis for the selective quantitation of an antigen in an unpurified extract which may contain structurally similar

molecules. The RIA is therefore typically more efficient than chromatographic methods because chromatographic analyses require an extensive purification of a sample. Losses of the analyte can occur during purification, and interfering compounds are not necessarily removed. The ability to use simple extracts provides substantial savings in processing times and material costs involved in the often extensive purification of samples. Furthermore, more samples can be processed simultaneously by RIA. This greater sampling capacity is necessary for analyses such as the screening of a population of clones for the selection of high producing variants.

CHAPTER II

MATERIALS AND METHODS

All chemicals and reagents were purchased from Sigma Chemical, St. Louis, MO, unless otherwise noted. The generous gifts of rare tropane alkaloids included 6-hydroxyhyoscyamine received from Dr. W.C. Griffin, Pharmacy Department, University of Queensland, St. Lucia, Queensland, Australia, and from Dr. W.C. Evans, Department of Pharmacy, The University, Nottingham, England, and littorine recieved from Dr. J.R. Cannon, School of Chemistry, The University of Western Australia, Nedlands, Australia.

Initiation and Maintenance of Callus Cultures

Seeds of Datura innoxia were obtained from Dr. L.R. Worthen, Department of Pharmacognosy, University of Rhode Island, and from Dr. O. Schieder, Max-Plank Institute for Plant Breeding, Koln, West Germany. Seeds from the latter were designated line H 328. This line was derived from a doubled haploid plant obtained by anther culture and is high yielding for scopolamine (0.29% DWT). In addition, seeds of tetraploid D. innoxia were obtained from Dr. M. Constantin, Botany Department, University of Tennessee, Knoxville. The D. stramonium seeds were obtained from Dr. Worthen.

Callus tissue was initiated and maintained on 1% agar slants of Gamborg's B5 medium (1970) supplemented with 1 mg/L 2,4-dichlorophenoxyacetic acid and 0.1 mg/L kinetin. Medium was prepared from the B5 salts containing sucrose (Gibco, Chagrin Falls, OH) and dispensed

20 ml per 25 X 150 mm test tube. Cultures were maintained in the dark at 25 deg C and subcultured at five week intervals.

Callus cultures were initiated from leaf disks (6 mm dia.) taken from surface sterilized, healthy, fully-developed leaves of greenhouse grown plants and placed directly on the medium. Cultures were established from eleven plants of D. innoxia H 328, from fourteen plants of D. innoxia from the University of Rhode Island, from six plants of D. stramonium, and from five plants of the tetraploid D. innoxia. Other cultures used in this study included several lines established by Dr. D. Dougall, Botany Department, University of Tennessee, Knoxville. These were derived from D. innoxia H 328 and from single plants of D. kymatocarpa, D. pruinosa, and D. lanosa grown in the greenhouse by Dr. E. Schilling, Botany Department, University of Tennessee, Knoxville.

Preparation of the Immunogens

A derivative of (-)-scopolamine prepared by the method of Weiler et al. (1981) was obtained from Dr. Dougall to use as the hapten for conjugation to carrier proteins. This derivative, nor-(-)-scopolamine-N-beta-propionic acid (Figure 1), was conjugated to human serum albumin (HSA) by both the mixed anhydride and the carbodiimide condensation methods described by Weiler et al. (1981). A second immunogen was prepared by conjugating the hapten to keyhole-limpet haemocyanin (KLH; Calbiochem-Behringer, La Jolla, CA) by the carbodiimide method only. Conjugate preparations were dialyzed for four days at 4 deg C against frequent changes of deionized H_2O. The conjugates were diluted with phosphate buffered saline (PBS; Daie and Wyse, 1982) to a final

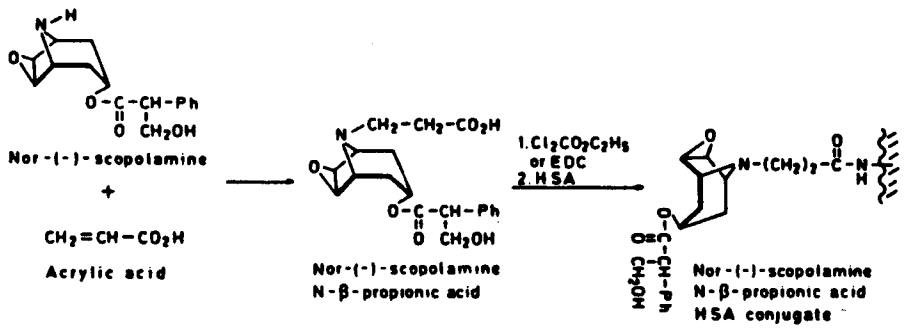


Figure 1. Synthesis of immunogen used for the preparation of scopolamine-binding antisera. Source: Weiler et. al. 1981. Phytochemistry Vol. 20, p. 2010.

concentration of 2 mg/ml, and stored frozen until needed for injection. Protein determinations were made with the BCA Protein Reagent (Pierce, Rockford, Il). The HSA preparations were pooled and partially denatured by heating to 60 deg C for 10 minutes before storage at -20 deg C.

Production of Antisera

Primary injections of immunogens were prepared by emulsification of 1 ml of immunogen stock into 1.5 ml of Freund's adjuvant (Difco, Detroit, MI). Half of the volume (containing 1 mg immunogen) was injected into a rabbit in four equal portions subcutaneously above each shoulder and intramuscularly in the hind legs. These injections were repeated twice at eight day intervals. The first and second of injections used Freund's complete adjuvant. The third set used a mixture of incomplete to complete (3:1) adjuvant. Half of a set of twelve rabbits was immunized with the KLH immunogen; the remainder received the HSA immunogen.

Booster injections of 0.5 mg immunogen emulsified in adjuvant were made after four weeks. The first contained an equal mixture of incomplete and complete adjuvant. The second injections were made eight days later and contained incomplete adjuvant only. Rabbits were bled via ear vein eight days after the final booster. The antisera were separated and treated with 0.5 mg sodium azide and with 0.009 ml phenol-ether (50:50 w/v) per ml of antiserum. Control antiserum was obtained from one rabbit by injecting HSA in place of hapten conjugated protein. The antisera were stored at -20 deg C.

Radioimmunoassay Procedure

The RIA was modified from the procedure of Weiler et al. (1981) and was performed by setting up a series of 12 X 75 mm disposable borosilicate glass culture tubes containing 0.4 ml of PBS + 0.25% BSA and 0.1 ml of (N-methyl[^{3}H])-scopolamine methylchloride stock solution. To a set of triplicate tubes for each scopolamine standard and sample extract, a volume of 0.1 ml was then added (these are B tubes containing competing unlabelled antigen). To another set of six replicates each, a volume of 0.1 ml PBS blank was added for T controls (total radioactivity of labelled antigen delivered to assay volume--incubated with non-immune serum) and for B_o controls (total radioactivity of labelled antigen bound by diluted antiserum). All tubes were mixed thoroughly with a vortex mixer and then 0.1 ml diluted antiserum was added to B and B_o tubes. Non-immune serum, diluted 1/125 was added to the T tubes. The tubes were mixed again and incubated at room temperature. After two hours an equal volume (0.7 ml) of 91% saturated solution of ammonium sulfate was added and, after mixing, the tubes were stored for 1 hour (or overnight) at 4 deg C. The tubes were then centrifuged at 3000 rpm (ca. 20 min.) to pellet the precipitate. A 1.0 ml portion of each of the supernatant fluids was transferred to 5.0 ml of Scintiverse II (Fisher Scientific, Fairlawn, NJ) in counting vials. The vials were then shaken or mixed vigorously with a vortex mixer to emulsify. Finally, the vials were stored in the dark for at least six hours before counting vials with a Beckman LS 3800 liquid scintillation spectrometer for at least two cycles of triplicate

counts. The counting error was ca. 1% when the maximum counting time was set for three minutes and a counting error set at 2 sigma.

Stock solutions of reagents used in the RIA were prepared ahead of time and stored frozen at -20 deg C, except for PBS buffers which were stored at 4 deg C. The stock solution of labelled antigen was prepared as 2.78 X 10^{-4} mCi/ml (3.5 X 10^{-12}moles/ml). One-tenth microliter of this gave approximately 14,000 cpm in the T control tubes. Antiserum was freshly prepared (from frozen aliquots) by dilution in PBS containing 0.25% BSA. Pig gamma-globulins (PGG) was added to the diluted antiserum and diluted non-immune serum to give a concentration of 10 mg/ml. (The dilution of antiserum that was be used in the assay was chosen by the prior determination of the dilution giving approximately 50% of the 14,000 cpm. This determination was made by a serial dilution of the antiserum and then testing each dilution as a series of B_o tubes.) The stock solutions of scopolamine standards were prepared by serial dilution in PBS. These solutions extended over the range of 0.6 to 30 ng/ml (at approximately 1/4 log intervals). The stock solution of ammonium sulfate was prepared as a 91% saturated solution (pH adjusted to 7.0).

Ethanol Extraction of Callus Tissue

Extracts of cultures were prepared for analysis by RIA in the following manner: Callus tissue was harvested during subculturing and then freeze-dried with a lyophilizer. A weighed sample (ca. 100 mg DWT) of tissue was then extracted overnight in 4 ml of EtOH + 28% NH_4OH (19:1). After centrifugation of the cellular debris, half of the

supernatant was removed, dried in vacuo, and then reconstituted with an equal volume of PBS (pH 7.4). The aqueous extracts were stored at 4 deg C overnight and centrifuged before assaying.

Solvent Partition Extraction of Callus Tissue for HPLC Analysis

A large bulk of callus tissue (ca. 300 g FWT) of D. stramonium was harvested from a single subculture and prepared by solvent partition extraction for HPLC analysis. The extraction involved packing about 5 grams of freeze-dried tissue into a cellulose thimble for Soxhlet extraction. The tissue was extracted overnight in hexane and then for 2 hours under reflux. The hexane was discarded and the tissue was allowed to dry. The tissue was then extracted with EtOH:28% NH_4OH (19:1) overnight and then under reflux for 6 hours. The ethanol extract was transferred to an evaporation flask, and the solvent was removed under reduced pressure. The residue was taken up in 0.1 N HCl (15 ml + 10 ml + 5 ml). The acidic phase was collected in a separatory funnel and washed twice with 50 ml volumes of $CHCl_3$. The aqueous phase was adjusted to pH 8.7 with KOH and then partitioned into $CHCl_3$ (three times with 50 ml). The $CHCl_3$ fractions were pooled, washed once with 50 ml H_2O and then transferred to an evaporation flask where the $CHCl_3$ was removed. The final residue for the total extracted tissue was taken up in 6 ml of 0.5% trifluoroacetic acid (TFA).

C_{18} Sep Pak Treatment of Samples

The sample prepared by solvent partition extraction was treated with a C_{18} Sep Pak (Waters Associates, Medford, MA) to further remove impurities from the sample. The C_{18} Sep Pak was pretreated, in order,

with 5 ml of 100% MeOH and 2 ml of 0.5% TFA, before loading standards or samples. Standards of scopolamine and hyoscyamine, or portions of the extract were loaded in 1 ml volumes of 0.5% TFA, and after the load volume was washed through, an additional 1 ml of 0.5% TFA was washed through. The alkaloids were completely eluted from the C_{18} Sep Pak with a wash of 50% MeOH (in 0.5% TFA). This was determined with an eluotrophic series of solvent washes in order of increased methanol content. Two ml solvent volumes were used and eluted completely before the next solvent volume was added. Each fraction was collected separately in a borosilicate glass culture tube.

HPLC Chromatography

A Waters Associates chromatograph which consisted of dual M-45 pumps, U6K Universal Injector, Model 441 Absorbance Detector operating at 229 nm, and a uBondapak C18 column (10 um packing; 3.9 mm x 30 cm column dimension) was used. A guard column was prepared with slurry-packed 37-50 um Bondapack C18 material. Eluted fractions were collected with an ISCO Model 1200 fraction collector. Chromatograms were prepared with a Houston Instruments Omniscribe recorder.

An isocratic mobile phase consisted of an aqueous solution of 0.2% phosphoric acid [adjusted to pH 7.25 with triethylamine (TEA)] and 15% MeOH. The aqueous and organic phases were separately filtered (0.25 um Millipore filter), mixed together, and the pH was readjusted to 7.25 before use. Chromatography was performed with continuous flow of the mobile phase at a rate of 1 ml/min. Alkaloid standards were injected before and after injections of samples. All solutions of

standards and samples were filtered (0.45 um) prior to injection. One ml fractions were collected at 1 min intervals for 40 min in borosilicate glass culture tubes, sealed with Parafilm, and stored at 4 deg C until assay by RIA.

CHAPTER III

RESULTS AND DISCUSSION

Establishment of the Radioimmunoassay

The RIA used here for the analysis of tissue cultures was set up as summarized here: Initially, an immunogen had to be synthesized that would elicit an immune response in rabbits. Two immunogens were synthesized by the procedure described by Weiler et al. (1981). Also, an antigen with a radioactive label had to be obtained for use in the RIA. (N-methyl[^{3}H])-scopolamine methylchloride was purchased for use as the labelled antgen. After immunization of rabbits with the immunogen preparations and collection of antisera, the antisera were screened to determine their titres for binding half of a limited quantity of labelled antigen. The antisera were again screened (at their 50% binding titres) to determine the ability of unlabelled scopolamine and hyoscyamine to compete with the labelled antigen.

Using the selected antiserum with an apparent high titre and sensitivity for scopolamine, the RIA procedure (Weiler et al., 1981) was modified so it could be routinely performed without the aid automated pipetting equipment. This required determining the conditions used for sampling the free antigen fraction of the reaction mixtures so that the amount of radioactivity bound by antiserum could be determined indirectly as the difference of the radioactivity in the supernatant fluids from the total redioactivity delivered to the assay mixtures. Under the conditions determined for sampling supernatant fluids, the amount of labelled antigen used in the assay was

readjusted, and then the antiserum dilution giving ca. 50% binding of the total radioactivity delivered to the assay mixture was redetermined. Once the final conditions for performing the RIA was established, the usable range of the scopolamine standard curve was determined. Finally, the cross-reactivity of the antiserum to related compounds was assessed. This assessment was made by preparing standard curves for each of the test compounds and comparing the mid-point of each with the mid-point of the scopolamine standard curve.

Screening of antisera. The first step in screening the antisera was the determination of the titres of each antiserum for binding (N-methyl[^{3}H])-scopolamine methylchloride. But prior to this, the quantity of the labelled antigen that would be used to test for binding had to be determined. This was done by taking 0.1 ml volumes of serial dilutions of the labelled antigen and adding, with 0.1 ml of H_2O, them to counting vials containing 5 ml of scintillation cocktail. the radioactivities in the vials were counted, and the dilution corresponding to 1.08×10^{-13} moles/ml labelled antigen was chosen for use in testing the antisera. Fifty percent binding (by antisera) of this quantity would give 10,000 cpm.

The antisera were serially diluted to 1/10,000 at approximate 1/3 log intervals (e.g. 1/5 to 1/10 to 1/20 . . . 1/10,000). The conditions for testing the antisera dilutions followed the procedure of Weiler et al. (1981). The counts per minute (cpm) bound in the precipitated antiserum (B_o) were determined as were the total cpm delivered to the assay mixture (T). The antiserum-dilution binding

curves were determined for each antiserum by the procedure described for antiserum KLH-10 (Figure 2). Non-immune serum was tested at 1/1 and 1/125 dilution and no specific binding of the labelled antigen was observed; non-specific binding by this control serum was ca. 1-2%. The 50% binding titres were estimated visually for each antiserum from their dilution binding curves. The titres estimated for each of the antisera are shown in Table 1. All rabbits responded to immunization, and titres for 50% B_o/T ranged from 1/5 to 1/200 (dilutions made prior to addition to the assay volume). Antisera obtained from the rabbits immunized the with HSA conjugate gave higher titres.

Those antisera with titres of 1/20 dilution or greater were then examined for the ability of authentic scopolamine and hyoscyamine to compete with the labelled antigen for antisera binding at the 50% binding titres. These tests were performed to determine the relative sensitivities between the antisera for scopolamine and also to obtain an estimation of the extent of cross-reactivity for hyoscyamine by the antisera. The results of these preliminary competition experiments are also shown in Table 1. Antiserum KLH-10, which had a good titre at a 1/100 dilution, was found to be the most sensitive for scopolamine competition: it gave a 50% B/B_o close to 1.08×10^{-12} moles/ml and was more selective for binding scopolamine than for hyoscyamine at this dilution. This antiserum was chosen for characterization and for the establishment of the RIA methodology. A second antiserum. HSA-12, was also chosen for characterization. (It provided a backup antiserum in case problems arose with the first antiserum.) Other antisera collected may also be suitable for use in the RIA, and they have been

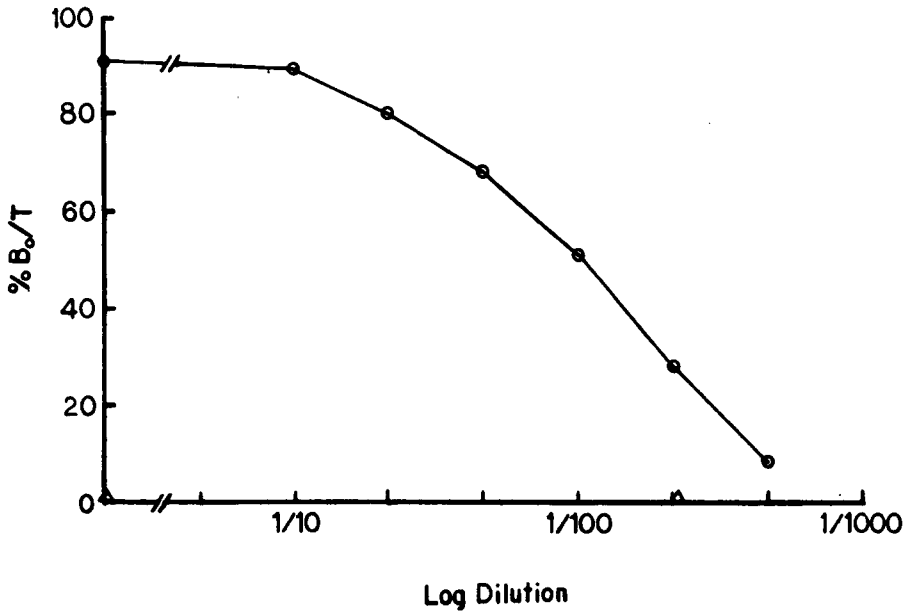


Figure 2. Antiserum-dilution binding curve for antiserum KLH-10. Each antiserum was tested to determine the titre giving 50% B_o/T. Tests were made by diluting the antisera at ca. 1/3 $\log_{10}$ intervals. One-hundred microliters of each dilution was added to 0.8 ml PBS + 0.25% BSA. After addition of 0.1 ml labelled antibody stock (ca. 20,000 cpm delivered) and incubation for one hour, the activity bound to antiserum was precipitated with 1 ml of 91 % saturated ammonium sulfate to give a final concentration of 46.5 %. After 30 min, the pellet was collected after centrifugation and quantitatively transferred in 0.2 ml H_2O to 5 ml scintillation cocktail in a counting vial. The cpm were determined for each dilution and the percent of total cpm delivered bound was plotted. Antiserum KLH-10 (○) and non-immunune serum (△). Non-immune serum was tested at 1/1 and 1/125 dilutions.

Table 1. Preliminary determination of antisera titres and ability of scopolamine and hyoscyamine to compete with labelled antigen.[a]

Serum #	Titre	Scopolamine[b]	%B/Bo	Hyoscyamine[b]	%B/Bo
KLH-1	1/5	Not examined		Not examined	
KLH-2	1/20	1.08×10^{-14}	106	1.08×10^{-14}	104
		" $\times 10^{-12}$	100	" $\times 10^{-12}$	110
		" $\times 10^{-10}$	52	" $\times 10^{-10}$	88
KLH-3	1/75	1.08×10^{-13}	89	1.08×10^{-13}	67
		" $\times 10^{-11}$	54	" $\times 10^{-11}$	62
		" $\times 10^{-9}$	12	" $\times 10^{-9}$	30
KLH-4	1/10	Not examined		Not examined	
HSA-5	1/200	1.08×10^{-14}	110	1.08×10^{-14}	105
		" $\times 10^{-12}$	107	" $\times 10^{-12}$	103
		" $\times 10^{-10}$	64	" $\times 10^{-10}$	77[c]
HSA-6	1/150	1.08×10^{-14}	108	1.08×10^{-14}	108
		" $\times 10^{-12}$	104	" $\times 10^{-12}$	111
		" $\times 10^{-10}$	68	" $\times 10^{-10}$	90
HSA-7	1/250	1.08×10^{-14}	101	1.08×10^{-14}	89[c]
		" $\times 10^{-12}$	101	" $\times 10^{-12}$	104
		" $\times 10^{-10}$	55	" $\times 10^{-10}$	73
HSA-8	1/100	1.08×10^{-13}	99	1.08×10^{-13}	81
		" $\times 10^{-11}$	60	" $\times 10^{-11}$	68
		" $\times 10^{-9}$	10	" $\times 10^{-9}$	31
KLH-9	1/5	Not examined		Not examined	
KLH-10	1/100	1.08×10^{-13}	74	1.08×10^{-13}	79
		" $\times 10^{-11}$	38	" $\times 10^{-11}$	75
		" $\times 10^{-9}$	0	" $\times 10^{-9}$	37
HSA-11	1/75	1.08×10^{-14}	89	1.08×10^{-14}	110
		" $\times 10^{-12}$	83	" $\times 10^{-12}$	104
		" $\times 10^{-10}$	44	" $\times 10^{-10}$	63

Table 1. (Continued)

Serum #	Titre	Scopolamine[b]	%B/Bo	Hyoscyamine[b]	%B/Bo
HSA-12	1/150	1.08 X 10^{-14}	96	1.08 X 10^{-14}	111
		" X 10^{-12}	92	" X 10^{-12}	114
		" X 10^{-10}	40	" X 10^{-10}	66

[a]The titres of antisera were determined at 50% B_o/T as described in Figure 2. Antigens were tested by adding 0.1 ml of standard dilutions to the 1 ml assay mixture containing antiserum diluted to the 50% B_o/T titre. Conditions of assay as described in Figure 2.

[b]Antigens were tested in duplicate tubes. Concentrations of standard solutions in moles/ml.

[c]Range of duplicates exceeded 10%.

stored frozen until their suitability in the RIA can be determined. Antiserum HSA-6 is also a potential candidate for use, it has a greater titre than KLH-10 and it may be more selective for scopolamine compared to hyoscyamine displacement. However, it appears to be less sensitive to scopolamine.

Modification of the radioimmunoassay. The competitive binding RIA is based on the distribution of the labelled antigen between the antiserum-bound and free fractions of an assay mixture. The RIA procedure described by Weiler et al. (1981) used the direct measurement of the radioactivity in the bound fraction. The antiserum in the assay mixture was precipitated, and after centrifugation, the supernatant fluid containing unbound antigen was decanted off. The precipitated fraction was then dissolved in a small volume of water and transferred to a counting vial to measure the radioactivity. This procedure was initially followed here but was soon found to be very tedious and time consuming. Furthermore, there was occasional spillage of the radioactive supernatant fluid during the decantation step. To simplify the procedure, and increase the number of samples that could be handled in an assay set, a portion of the supernatant fluids were transferred to the counting vials after the initial centrifugation step. The amount of radioactivity bound in the precipitated antisera was then determined indirectly by calculating the difference of the radioactivity measured in the supernatant fluids of standards and samples from the radioactivity measured in the supernatant fluids of the controls receiving no antiserum.

The separation of the bound fraction was found to be enhanced by the addition of 10 mg/ml of pig gamma-globulins to the antiserum reagent stock (Desbuquois and Aurbach, 1971). This protein reduced the standard deviation of triplicates to ca. 2% of B_o by acting as a "carrier" during precipitation and centrifugation of the bound fraction.

The measurement of radioactivity in the supernatant fluids was optimized by reducing the volume of the assay mixture to 0.7 ml. After the addition of the equal volume of ammonium sulfate, a 1 ml volume (which contained the majority of the supernatant fluid) could be transferred without disturbing the centrifuged precipitate. After this modification was established as reproducible, the amount of labelled antigen was changed so that ca. 14,000 cpm would be contained in the T control tubes (the radioactivity delivered to tubes containing non-immune serum). The counts per minute transferred from the B_o control tubes was ca. 7000 cpm (when 50% of the radioactivity delivered to the assay was bound), and the range of radioactivity transferred from the scopolamine standards was roughly 8,000 - 13,000 cpm.

Scopolamine standard curves. Typical standard curves for scopolamine obtained for antisera KLH-10 and HSA-12 under the conditions established are shown in Table 2. These standard curves represent combined data from three separate repetitions of the RIA for both antisera. The dilutions of the antisera stocks were 1/250 for KLH-10 and 1/400 for HSA-12. At these dilutions binding of the total activity delivered is ca. 50% for KLH-10 and ca. 65% for HSA-12. The standard curves for the two antisera are very similar. They both have

Table 2. Representative scopolamine standard curves for antisera KLH-10 and HSA-12.

scopol. eqs.	$\bar{Y}^a$	$Y_{calc}{}^b$	$(\bar{Y}-Y_{calc})^2$
	Antiserum KLH-10		
1.5 ng/ml	79.44	78.44	1.0000
3	62.96	64.14	1.3924
6	51.42	49.84	2.4964
10	38.22	39.30	1.1664
15	31.40	30.39	0.2209
Corr. coeff.= -0.974			SE = 1.253
Y' (b) = 86.81%			CV (50% Y)= 2.51%
Slope (m) =-47.51			
X' = 67.18 ng/ml			
50% B/B_o = 5.95 ng/ml			
= 19.6 pmol/ml			
	Antiserum HSA-12		
1.184 ng/ml	83.99	89.39	12.960
1.5	83.99	83.78	0.0441
2.368	71.40	72.96	2.4336
3	69.72	67.36	5.5960
4.735	58.19	56.54	2.7225
6	53.74	50.92	7.9524
7.892	42.64	44.43	3.2041
10	35.65	38.82	10.048
11.84	33.66	34.82	1.3456
Corr. coeff.= -0.9784			SE = 2.406
Y' (b) = 93.39%			CV (50% Y)= 4.81
Slope (m) =-54.57			
X' = 51.45 ng/ml			
50% B/B_o = 6.24 ng/ml			
= 20.6 pmol/ml			

[a] $\bar{Y}$ represents mean of $\%B/B_o$ from three assays.
[b] Y_{calc} is value determined from least squares method.

a linear response over the approximate range of 1.5 to 25 ng/ml when B/B_0 is plotted against log scopolamine eqs. ng/ml. The midpoint for both is near 6 ng/ml and the limit of detection is about 0.6 ng/ml.

A summary of RIA performance markers for the assays carried out under standard conditions is given in the Table 3. These markers are measurements such as the total activity bound (B_0/T) or midpoint of the standard curve (50% B/B_0) determined for each assay and are used to monitor the reproduction of the standard curves. These assays were performed under constant conditions as described in MATERIALS AND METHODS and the standard curves have been satisfactorily reproduced. This reproducibility includes assays performed with different preparations of reagent stocks and buffers.

Sensitivity of the radioimmunoassay. The sensitivity of the competitive binding RIA is determined by the avidity of the antiserum and the specific radioactivity of the labelled antigen (Landon and Moffat, 1976). The avidity of the (polyclonal) antiserum is determined by the relative proportions of different populations of antibodies that exhibit different affinities (binding constants) for the antigen. This RIA used the commercially prepared (N-methyl[^{3}H])-scopolamine methyl chloride as the labelled antigen in the assay. The data indicated the two antisera examined have a high avidity for this compound based on the titres of the antisera and sharp slope of the scopolamine standard curve. These results are comparable to the scopolamine RIA described by Weiler et al. (1981) and for other hapten RIAs (Robbins, 1986).

Table 3. Summary of RIA performance markers for the scopolamine standard curve.[a]

Date	T	Bo	%Bo/T	Mid-point	Slope
		Antiserum KLH-10			
Mar 31[b]	15469±683	9089±325	58.76±3.58	5.96	-49.08
May 08	14667±266	8007	54.60	5.40	-43.90
May 15	14918±312	7871±118	52.80±1.50	N.D.[c]	N.D.
May 23	15439±374	5379±112	34.84±2.07	6.21	-46.64
Jun 13	15108±286	7870±224	52.09±2.89	N.D.	N.D.
Jul 02	15339±189	7624±099	49.70±1.30	N.D.	N.D.
Jul 09	13517±337	7263±102	53.73±1.40	4.54	-52.18
Jul 23	14949±289	7678±160	51.36±2.10	5.60	-47.38
Spt 18	14615±254	8087±283	55.33±3.50	4.24	-50.39
Oct 13[d]	10646	6051	56.80	5.22	-50.27
Oct 21	14565	6683±251	45.89±3.76	5.87	-51.31
Nov 11	15051±896	7869±311	52.28±3.95	4.36	-51.03
Nov 25[e]	13537±176	4770±139	35.24±2.92	5.83	-56.43
Dec 05[e]	13928±128	4903±086	35.12±1.75	7.62	-50.36
Jan 16	15438±262	6640±202	43.01±3.04	N.D.	N.D.
Jan 23	14415±345	8095±191	56.16±2.37	4.36	-51.02
Mar 18	13527±342	7231±212	53.45±2.53	2.18	-35.12
Mar 30	14115±167	6810±067	48.25±1.01	2.42	-29.60
Apr 04	14451±112	8213±175	53.64±2.14	4.69	-49.69
Apr 17	14853±221	8622±104	58.05±1.21	N.D.	N.D.
May 01	14293±172	7521±123	52.62±1.64	2.71	-46.62
		Antiserum KLH-12			
Apr 12[b]	15936	11323±356	71.06±3.14	6.05	-52.12
Apr 29	12911±305	8684±134	67.26±1.56	6.0	N.D.
May 20	15142±731	8108±267	53.55±2.62	N.D.	N.D.
Jul 16	14057	9833±133	69.95±1.35	6.03	-55.09
Spt 18	15542±233	10265±108	66.05±1.05	6.51	-52.01
Jan 14	15229±325	8936±096	58.68±1.52	N.D.	N.D.
Mar 18	13029±171	8855±089	67.97±1.01	7.76	-40.27
Apr 01	14163±271	9135±142	64.50±1.55	6.00	-39.44
Apr 04	14415±112	9521±109	66.05±1.15	6.42	-50.97

[a]Assays performed under established conditions as described in MATERIALS AND METHODS.
[b]A 1 ml assay mixture volume was used on this date.
[c]N.D.: not determined.
[d]A 1 ml volume of ammonium sulfate was used on this date.
[e]The antiserum was used as a 1/300 dilution on this date.

Weiler et al. (1981) concluded (N-methyl[^{3}H])-scopolamine methylchloride was unsuitable for use in the RIA because their antiserum had a low affinity for this compound. In their RIA (N-methyl[^{3}H])-scopolamine methylchloride was readily displaced by other tropane alkaloids in addition to scopolamine and the RIA was consequently nonspecific for scopolamine when methylscopolamine was used as the labelled antigen. Weiler et al. (1981) decided to synthesize (N-C^{3}H$_3$)-scopolamine for use as labelled antigen in their RIA. The specific activity of this compound was ca. 100-fold less than tritiated methylscopolamine. In contrast both antisera described here demonstrated ready displacement of radiolabelled (N-methyl[^{3}H])-scopolamine methylchloride by scopolamine without loss of specificity against other tropane alkaloids (see next section). Considerable time and effort was thereby saved by not having to synthesize a labelled antigen. The ability to use tritiated methylscopolamine with the antisera described here resulted in an approximate 10-fold increase in the sensitivity of the assay range of this RIA. This sensitivity can be increased further, but the changes in the assay necessary for this would result in a less precise assay that would be more difficult to routinely perform.

Characterization of antisera specificity. A competitive RIA for quantitating scopolamine yields in culture extracts requires an antiserum that is highly specific for binding scopolamine. A high specificity is necessary because other tropane alkaloids of closely similar structure are likely to be present in the extracts. These can

potentially be bound by the antisera and thereby increase the apparent yields of scopolamine by competing with the labelled antigen for antibody binding sites.

The specificities of antisera KLH-10 and HSA-12 were characterized by determining the extent that scopolamine-related alkaloids displace the labelled antigen in the assay. This displacement was measured as the percent cross-reaction of the compounds relative to scopolamine displacement. The cross-reactivity is defined as the molar quantity of the test compound displacing 50% of total activity bound divided by the molar quantity of scopolamine displacing 50% of the total activity bound (Weiler and Zenk, 1976). Serial dilutions of the test compounds were prepared in the manner of scopolamine standards, and the molar quantities displacing 50% of total activity bound was calculated from standard curves derived for each of the test compounds.

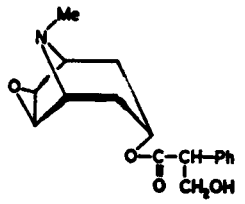
The cross-reactivities determined for the related alkaloids obtained for analysis are shown in Table 4. Determinations were also made for the hapten conjugate and certain other synthetic derivatives of scopolamine. The structures of these antigens are shown in Figure 3. As indicated here and from the representative scopolamine standard curves, the antisera appear to be equally sensitive to displacement of (N-methyl[^{3}H])-scopolamine methylchloride by scopolamine, although the titre of HSA-12 is nearly twice that of KLH-10. However, the two antisera differed significantly in their specificities for the other tropane alkaloids examined with KLH-10 being more specific than HSA-12.

Two compounds of particular interest are 6-hydroxyhyoscyamine and hyoscyamine. These two are the immediate biosynthetic precursors to

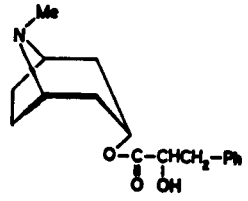
Table 4. Cross-reactivities of antisera KLH-10 and HSA-12.[a]

Antigen	Moles/ml= 50% B/Bo	%CR
	Antiserum KLH-10	
scopolamine	1.96×10^{-11}	100
hyoscyamine	3.78×10^{-10}	5.19
6-hydroxyhyoscyamine	1.19×10^{-9}	1.65
norscopolamine	9.47×10^{-11}	20.7
scopine	1.50×10^{-7}	0.01
tropine	$>1.0 \times 10^{-6}$	>0.000
tropic acid	$>1.0 \times 10^{-6}$	>0.000
homatropine	$>1.0 \times 10^{-6}$	>0.000
littorine	1.67×10^{-6}	0.001
atropine	3.15×10^{-10}	6.23
norscopolamine-N-propionic acid	1.88×10^{-12}	1043
methylscopolamine	3.60×10^{-12}	544
	Antiserum HSA-12	
scopolamine	2.06×10^{-11}	100
hyoscyamine	3.08×10^{-11}	66.9
6-hydroxyhyoscyamine	3.11×10^{-10}	6.62
norscopolamine	1.75×10^{-10}	11.77
scopine	1.41×10^{-7}	0.01
tropine	$>3.0 \times 10^{-6}$	0.00
tropic acid	$>3.0 \times 10^{-6}$	0.00
homatropine	9.12×10^{-8}	0.02
littorine	$>1.0 \times 10^{-7}$	<0.02
atropine	4.95×10^{-11}	41.6
norscopolamine-N-propionic acid	1.70×10^{-11}	121
methylscopolamine	4.82×10^{-12}	427

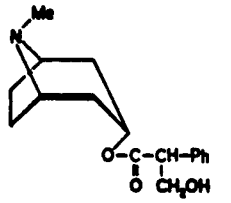
[a]Standard curves were prepared for each test compound and the concentration giving 50% B/B_o was used to determine the percent cross-reactivitiy relative to the concentration of scopolamine giving 50% B/B_o.



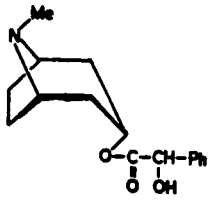
Scopolamine



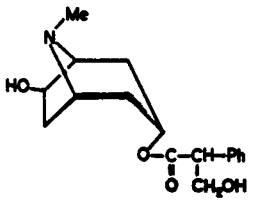
Littorine



Hyoscyamine

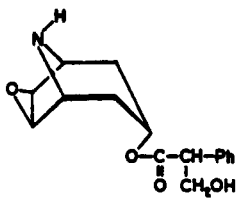


Homatropine*

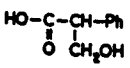


6-Hydroxyhyoscyamine

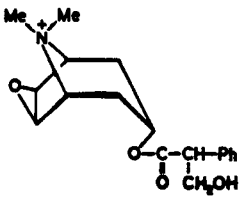
Tropine



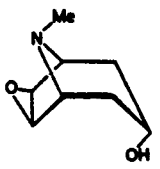
Norscopolamine



Tropic acid



N-methylscopolamine*



Scopine

Figure 3. Structures of natural and synthetic(*) tropane alkaloids examined by RIA.

scopolamine (Hashimoto and Yamada, 1986) and differ only at the 6,7-oxy-substituent (Figure 3). This position is close to the site of conjugation and its contribution as an antigenic determinant (epitope) is thereby reduced. Hyoscyamine was found to be 5.19% cross-reactive with KLH-10 which was 13-times less cross-reactive than with HSA-12. The cross-reactivity of 6-hydroxyhyoscyamine by KLH-10 was 1.65% and this was 4-times less cross-reactive than with HSA-12. The specificities exhibited by KLH-10 for these two biosynthetic precursors of scopolamine is comparable to the scopolamine-specific antiserum first described by Weiler et al. (1981); this is not unexpected since the same site of conjugation was used here. Because hyoscyamine is known to be produced in excess of scopolamine in callus tissue cultures of Datura (Hiraoka and Tabata, 1974; Netien and Combet, 1966), the low cross-reactivity exhibited by antiserum KLH-10 for hyoscyamine may be sufficient to compromise the validity of the scopolamine determination in simple extracts of callus tissue using this antiserum.

The highest specificities, by both antisera, were directed toward those structures furthest away from the site of conjugation. Extreme decreases in cross-reactivity (1×10^5 for KLH-10 and 4×10^3 for HSA-12) occured with alterations in the tropic acid moiety, e.g. compare hyoscyamine to its isomer littorine or to homatropine (a synthetic scopolamine derivative). Products formed by the hydrolysis of the ester linkage, viz. scopine, tropine, and tropic acid, were at least 1×10^4 less cross-reactive compared to scopolamine. This

indicates the ester-linkaged tropic acid moiety is required for antiserum recognition.

The cross-reactivity of atropine was also examined. Atropine is racemic hyoscyamine formed during the extraction process. The differences of cross-reactivity by both antisera between these two compounds is small, so the natural (-) conformation of the tropic acid moiety does not confer much specificity for their binding by the antiserum.

The hapten conjugate, norscopolamine-beta-propionic acid, and methylscopolamine are synthetic N-substituent derivatives and demonstrated greater reactivities (10-fold and 5.4-fold, respectively) than scopolamine by KLH-10. Antiserum HSA-12 did not differentiate between the hapten conjugate and scopolamine greatly, but the antiserum still exhibited a preference for methylscopolamine (4.3-fold greater). The removal of the methyl group from the N-substituent of scopolamine (giving norscopolamine) demonstrated a decreased cross-reactivity by both antisera (21% and 12% for KLH-10 and HSA-12, respectively).

Norhyoscyamine and the N-oxides and apo-derivatives of scopolamine and hyoscyamine are other tropane alkaloids that have been isolated from plant tissues (Evans, 1979), but their presence in tissue cultures have not been reported. These compounds were not available for analysis, but based on the nature of the cross-reactivities of those standards examined, norhyoscyamine and apo-derivatives would not be expected to be significantly cross-reactive. The determination of cross-reactivity by N-oxides would be interesting for comparison to other N-moiety derivatives.

The highest specificities of an antiserum are directed toward those structures of a hapten furthest from the site of conjugation (Landon and Moffat, 1976), and this was seen here. Antisera of higher specificity for the epoxide group would likely have been obtained if scopolamine was conjugated via a structure in the tropic acid moiety. Conjugation of scopolamine to the carrier protein at the tropane nitrogen was used in this study because this method was successful for Weiler et al. (1981) in eliciting scopolamine binding antisera. Weiler et al. (1981) also attempted glutaric anhydride coupling through the hydroxyl side group of tropic acid, but no anti-hapten activity was detected in any of the rabbits injected with this immunogen. They probably did not get the desired conjugation because of the complete lack of immunogenic response in any of the rabbits, but this is not known for certain because they did not mention whether or not they tested this immunogen to confirm hapten conjugation.

A second site for scopolamine conjugation in the tropic acid moiety that could potentially be used to obtain antisera with a high specificity at the tropane moiety is the coupling via the phenyl ring with diazotized p-aminobenzoic acid. Virtanen et al. (1980) used this to obtain antisera specific for atropine and l-hyoscyamine with a cross-reactivity for scopolamine less than 0.1%. Similar specificities, but reversed, can potentially be obtained by substituting scopolamine for hyoscyamine for immunogen synthesis.

Radioimmunoassay of Ethanol Extracts of Callus Tissue

Simple callus extracts were included in the assay sets as internal standards to assess the reproducibility of RIA determinations of samples and to determine if the dilution of samples was linear in the useable range of labelled antigen displacement. The RIA was also applied to extracts of all culture lines to determine if the were producing any antigenic activity, and if they were, then the relative levels of activitity production between cultures could be assessed. Finally, the consistancy of level of production could be monitored. In all applications antigenic activity is determined as scopolamine equivalents, unless the activity was specifically determined.

Analysis of standard ethanol extracts. Two test samples were prepared by pooling ethanol extracts derived from D. stramonium callus cultures. These samples were used as internal standards to make an assessment of how reproducible the measurement of yields are between different performances of the RIA. The results from four consecutive assays of the samples, for both antisera, are shown in Table 5. An estimate of the between-assay error, determined as the coefficient of variation (CV) of extract yields, is 6.77% with the range 3.11 to 12.02%. These results are comparable to those reported for the RIA of other secondary products (Weiler, 1977).

The data in Table 5 also show yields of scopolamine equivalents increased 3-fold when the extracts were assayed with the less specific HSA-12 antiserum. Both antisera have approximately the same reactivity towards scopolamine (Table 2, p. 30). The increase in antigenic activity

Table 5. Four repetitions of the RIA of two ethanolic extracts of D. stramonium callus.

	%B/Bo	ng/ml		%B/Bo	ng/ml
			Antiserum KLH-10		
#1	40.51±8.48	9.43	2#	60.18±5.47	3.64
	40.79±2.46	9.30		58.72±3.46	3.90
	37.22±2.33	11.06		61.72±5.05	3.37
	35.79±0.93	11.85		60.46±0.59	3.59
	38.58	10.41±1.25 (12.0%)[a]		60.27	3.53±0.14 (3.91%)[a]
			Antiserum HSA-12		
#1	12.77±0.77	30.02	#2	29.36±2.66	14.91
	12.49±3.09	30.37		27.22±0.51	16.31
	13.10±2.50	29.60		24.96±1.80	18.10
	11.42±1.07	31.78		26.28±0.93	16.97
	12.43	30.44±0.95 (3.11%)[a]		26.91	16.57±0.33 (8.04%)[a]

[a]Coefficient of variation of the mean yield for four assays of the extract.

of the extract when measured by the generally less specific antiserum HSA-12 can be attributed to the presence of an antigenic component(s) for which HSA-12 is more sensitive. The identity of the antigenic component(s) cannot be inferred from this data, nor can the presence of scopolamine in the extract be confirmed by this data.

A third ethanolic extract derived from D. stramonium callus tissue was prepared, and serial 2-fold dilutions were made to determine the linearity of the measured activity of the dilutions in the range of 20-80% binding of activity. Serial dilutions were assayed with both antisera and the results are shown in Table 6. The displacement of labelled antigen by the extract showed a linear response to dilution with both antisera. These results show the assay of these extracts is not effected by the extent of dilution (as long as the extract is diluted to a concentration of antigen activity falling within the linear response range of the standard curve).

Measurement of yields of Datura callus cultures. Callus tissue cultures have been established from leaf tissue explants for five species of Datura. Ethanol extracts were prepared for all culture lines and were subjected to a preliminary analysis for their scopolamine yields. These lines represented 42 different greenhouse grown plants. Displacement activity was detected in all cultures examined, and the D. stramonium lines exhibited the highest yields (data not shown). From this analysis ten lines were selected for their apparent high "scopolamine" yields for continued maintenance as

Table 6. Evaluation of linearity of displacement of the labelled antigen by a serially diluted ethanol extract of *D. stramonium* callus.

Dilution	%B/Bo	ng/ml[a]	CV%
	Antiserum KLH-10		
1/10	23.94±4.58	217.7±39.2	48.1
1/20	37.48±2.99	245.2±31.6	12.9
1/40	52.66±2.75	259.8±29.2	11.2
1/80	70.52±0.40	245.8±08.0	3.3
1/160	84.99±2.60	268.8±23.0	8.61
Slope of dilution line = -51.53			
	Antiserum HSA-12		
1/1	25.23±4.76	23.20±5.26	22.68
1/2	38.41±1.97	24.37±2.26	9.28
1/4	52.00±2.52	25.40±3.09	12.15
1/8	65.77±2.40	26.17±2.97	11.36
1/16	80.29±1.26	25.96±1.58	6.10
Slope of dilution line = -45.79			

[a]Antigenic activity yield in scopolamine eqivalents with correction for dilution. Standard deviation is for triplicate tubes.

stock cultures. Cultures retained include lines representing each of the five species.

This initial screening of the culture stocks was performed with an uncharacterized antiserum whose quality could not be fully assessed. The screening of the culture stocks with this antiserum was necessary to reduce large number of cultures that were being maintained. All subsequent assays were performed with antiserum KLH-10 unless otherwise noted. Furthermore, yields of extracts are given as scopolamine equivalents since the quality and quantity of antigenic components in extracts are unknown.

Measurements of the yields by antiserum KLH-10 of the selected cultures and three additional species are given in Table 7. Yields of antigenic activity, determined as scopolamine equivalents, ranged from a high of 3.7 ug/g DWT for D. stramonium line #833-16-2 to a low of 19 ng/g DWT for D. innoxia line H328-2. Fluctuations in the activity yields were observed over time when these two and other cultures were re-assayed at intervals. D. stramonium again demonstrated the highest average yields for all species of Datura maintained as callus cultures. An ethanol extraction was made of leaf tissue taken from a plant of D. innoxia and from D. stramonium for comparison of yields to callus tissue. Antigenic activity obtained from this differentiated tissue was measured as 239 mg/g DWT and 942 mg/g DWT for D. innoxia and D. stramonium, respectively. Yields of activity in callus tissue is about 1000-fold less than the yields of differentiated leaf tissue. This apparent low production of scopolamine by undifferentiated cultures is consistent with results of others who have grown Datura

Table 7. Measurements of antigen activity of callus cultures.[a]

Culture line	Species	Yield[b]	Time in culture
#833-16-2	D. stramonium	3.7	26 months
		0.73	36 "
#833-17-2	" "	0.75	26 "
#833-18A2	" "	0.71	26 "
#833-19-2	" "	0.53	26 "
		0.31	36 "
		0.40	38 "
		0.55	40 "
#833-13C	D. innoxia	0.053	26 "
		0.73	36 "
H 328-2	" "	0.24	6 "
		0.019	12 "
		0.033	24 "
H 328-D	" "	0.13	12 "
		0.09	24 "
# 879-N1	" "	0.46	6 "
		0.25	10 "
		0.13	16 "
# 879-N6	" "	0.16	6 "
		0.11	10 "
		0.064	16 "
D L	D. lanosa	0.22	6 "
		0.084	16 "
		0.21	18 "
D P	D. pruinosa	0.16	6 "
		0.035	16 "
		0.03	18 "
D K	D. kymatocarpa	0.22	6 "
		0.05	16 "
		0.095	18 "
#6875 Ad-1	D. innoxia	0.021	---

[a]RIA of ethanolic extracts with antiserum KLH-10.
[b]Yields are given as ug scopolamine eqs./g DWT.

spp. in culture (Hiraoka and Tabata, 1974; Netien and Combet, 1966; Chan and Staba, 1965).

Variations in yield were exhibited between cultures derived from different individual plants of the same species and from cultures derived from the same original explant. This variation was examined more closely with D. stramonium cultures. As shown in Table 8 differences in yields were observed in culture lines derived from four different plants and also from sublines derived from the same explant. Sublines derived from the same explant taken from plant #16 gave the highest average yields for this species. Subline #16-1 measured twice the activity in #16-4 and was ca. 10-fold greater than some sublines derived from other plants.

From this comparative analysis of the D. stramonium sublines, line #16 was selected for expansion to collect a large quantity of tissue for a later bulk solvent extraction and purification to be used for the separation of its antigenic components by HPLC.

Effects of growth regulators on yields of callus tissue. Yields of secondary products in plant cell cultures are strongly affected by both the quality and the quantity of the growth regulators present in the medium (Zenk et al., 1975; Dougall, 1979). A preliminary examination was made here to determine the effect of the deletion of the growth regulators on the yield of antigenic activity in the cultures. Callus tissue from four lines, two each derived from different plants of D. innoxia and D. stramonium, were subcultured onto B5 medium containing four combinations of deletion of the standard

Table 8. Analysis of D. stramonium callus cultures by antiserum KLH-10.

Subline #[a]	%B/Bo	Yield[b]
16-1	40.68±2.28	3.13
16-2	47.91±1.29	2.29
16-3	48.17±0.83	2.29
16-4	52.80±1.55	1.51
17-1	85.10±0.56	<0.39
17-2	96.44±2.13	<0.33
17-3	81.57±1.19	0.47
17-4	91.03±0.39	<0.33
18A-1	98.71±1.08	<0.33
18A-2	89.14±1.57	<0.33
18B-1	91.21±1.29	<0.33
18B-2	92.00±2.24	<0.33
18B-3	70.66±2.12	0.81
19-1	63.97±2.90	0.96
19-2	96.44±2.30	<0.33
19-3	76.42±2.61	0.55
19-4	94.10	<0.33

[a]Four sublines derived from single explant taken from plant #16, 17, and 19. Two explants from plant #18, A and B, from which two and three sublines were derived, respectively.

[b]Yields of antigenic activity are given in ug scopolamine eqs./g DWT.

supplementation of the growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin. The yields of the cultures in scopolamine equivalents (as determined by antiserum KLH-10) of the tissue grown under the combinations of the growth regulators is shown in Table 9.

The deletion of 2,4-D from the growth medium resulted in the nearly complete cessation of callus growth for all lines examined. The exclusion of kinetin did not appear to effect growth in any of the lines. Tissue of D. innoxia lines grown on medium without the supplemented 2,4-D produced higher yields of antigenic activity. However, the D. stamonium lines produced higher yields when grown on the medium containing 2,4-D. No pattern of an effect on the production of antigenic activity for either species was evident by the presence or absence of kinetin in the medium.

These results indicate the yields of Datura may be affected by the auxin contained in the growth medium, but this effect needs to be verified statistically. This cursory study provides a basis for a more thorough investigation concerning the auxin source in order to obtain higher productivities by the cultures.

HPLC Analysis of D. stramonium Callus Tissue

The demonstration of an increase in the yield of a D. stramonium callus culture extract when the extract was assayed with the less specific antiserum HSA-12 indicated the presence of antigenic components other than scopolamine. Although this was anticipated, particularly for hyoscyamine, the extent that cross-reacting compounds are produced in callus cultures is not known. If other compounds are

Table 9. Effects of growth regulators on alkaloid yields.

Culture #	Medium	Yield±SD[a]	CV%[b]
D. innoxia:			
#879-N6	B5	1.12±0.03	2.35
	B5+D	0.07±0.01	9.35
	B5+K	>1.57±0.63	4.01
	B5+D+K	0.11±0.01	7.63
#879-N1	B5	9.25±0.87	9.41
	B5+D	0.14±0.01	10.33
	B5+K	7.72±0.15	1.94
	B6+D+K	0.25±0.02	9.20
#843-D-1	B5	0.17±0.02	12.1
	B5+D	0.05±0.00	8.00
	B5+K	>0.36±0.02	5.98
	B5+D+K	<0.07	----
D. stramonium:			
#833-16-1	B5	0.77±0.03	4.15
	B5+D	1.97±0.24	12.2
	B5+K	0.60±0.06	10.1
	B5+D+K	1.91±0.07	3.66
#833-19-2	B5	0.54±0.03	5.56
	B5+D	1.19±0.07	5.88
	B5+K	0.65±0.05	7.42
	B5+D+K	0.55±0.07	12.73

[a]Yields of antigenic activity given as ug scopolamine equivalents/g DWT.

[b]Coefficient of variation of the mean yield for triplicate tubes.

produced and are present in quantities in excess of scopolamine, then antiserum KLH-10 cannot be used to specifically quantitate scopolamine in simple ethanolic extracts of callus cultures. The purpose of this third area of experimentation, then, was to determine if cross-reacting compounds are produced in callus tissue in sufficient quantities such that the antiserum KLH-10 cannot selectively measure scopolamine in simple extracts.

This determination was made by extraction of ca. 15 g DWT of callus tissue of D. stramonium by solvent partition extraction, preparation of the sample by C_{18} Sep Pak treatment, separation of antigenic components by HPLC reverse phase chromatography, and then determination by RIA the extent other antigenic components contribute to the apparent yield of the extract.

Preparation of a Datura stramonium extract. Callus tissue (16.3 g dry weight) of D. stramonium line #16 was collected and extracted by the solvent partition extraction method described in MATERIALS AND METHODS. The final extract, DS16E1, was taken up and stored in 6 ml of 0.5% TFA. Recoveries of yield were monitored by RIA at three steps of the extraction process (Table 10) and were determined as a percentage of antigenic activity of the simple ethanolic extract. A quantity of 50 ug of authentic scopolamine and hyoscyamine (1:1 mixture) was added to EtOH:NH_3OH and worked up in parallel with the DS16E1 extraction to measure the recovery of the alkaloids during the solvent partitioning. The total antigenic activity recovered for DS16E1 was ca. 29 ug scopolamine eqs as measured by antiserum KLH-10 (87 ug by HSA-12).

Table 10. Activity recovered during solvent partition extraction of DS16E1.[a]

Phase	DS16E1[b]	Control[c]
HCl	85.9%	78.7%
$CHCl_3$	68.5	77.8
Final	57.5	72.3

[a]Antigenic activity measured with antiserum KLH-10.

[b]Expressed as percentage of activity in the initial ethanol extract.

[c]50 ug scopolamine and hyoscyamine (1:1) added to EtOH:NH_3OH and worked up in parallel to measure recovery of authentic compounds during the solvent partitioning.

The solvent partition extraction gave a yield of 1.78 ug scopolamine eqs./g DWT for D. stramonium line #16 by antiserum KLH-10.

Preliminary experiments were performed to determine conditions for adsorbing and eluting scopolamine and hyoscyamine from a C_{18} Sep Pak. This treatment was necessary to selectively remove impurities from the DS16E1 extract, particularly the very hydrophobic components which can cause deterioration of the performance of the C_{18} HPLC column.

For the initial determination of elution conditions 5 ug and 50 ug quantities of a mixture of authentic scopolamine plus hyoscyamine (1:1) were loaded and the antigenic activity was eluted with a solvent series of 0.5% TFA containing increasing proportions of methanol. The eluted fractions were assayed with antiserum HSA-12. The distribution of the activity eluted from the C_{18} Sep Pak for both sets are shown in in Table 11. The alkaloids were eluted over the 20, 30, and 40% MeOH fractions, and 100% of the activity loaded was recovered.

The results of the elution of scopolamine and hyoscyamine standards indicated a selective elution of these compounds in the DS16E1 extract could be achieved by a single elution with 50% MeOH. Prior to this elution a wash with 5% MeOH would be used to remove the more polar contents of the sample. The more hydrophobic components of the extract would be retained on the C_{18} Sep Pak. This procedure was followed by loading 50 ul and 250 ul volumes of DS16E1 onto C_{18} Sep Paks and then eluting with 2 ml portions of 0% (the load volume), 5, 50, and 100% MeOH in sequence. The fractions collected were assayed with antiserum KLH-10, and the antigenic activity in each of the

Table 11. Determination of solvent strength for the elution of authentic scopolamine and hyoscyamine from a C_{18} Sep Pak.

MeOH[a]	%B/Bo	ng/ml	%B/Bo	ng/ml
	5 ug load		50 ug load	
0%	101.4	-0-	98.3±3.7	-0-
5%	98.6	-0-	98.1±1.1	-0-
10%	92.5	0.0	67.7±2.9	3.0
20%	36.0	15.4	21.9±0.7	22.6
30%	6.1	>>57.4	17.1±0.5	28.0
40%	16.1	>37.1	28.9±2.5	16.5
50%	67.7	3.8	79.7±0.3	1.7
75%	99.6	-0-	97.0±0.6	-0-
100%	100.3	-0-	100.2±1.1	-0-

[a]All solvents except 100% MeOH contain 0.5% TFA.

fractions is shown in Table 12. The activity loaded was completely bound, and ca. 92% of the loaded activity was recovered in the two volumes of 50% MeOH.

Determination of HPLC solvent composition. A reverse phase chromatography mode described by Plank and Wagner (1986) for the separation of scopolamine and hyoscyamine extracted from D. innoxia plant tissue was modified for use here. This required the determination of a mobile phase composition that provide an efficient separation of scoplamine from the tropane alkaloids likely to be present in the extract. The standards, scopolamine, hyoscyamine, 6-hydroxyhyoscyamine, and norscopolamine, were injected in series and in combinations, and their retentions times were determined at different solvent strengths of the mobile phase. The solvents differed by 5% increments from 10% to 35% MeOH. From these data a 15% MeOH content was selected as giving satisfactory separation of the standards (Figure 4). A Waters Model 660 Solvent Programmer was used to make the changes in the methanol content of the mobile phase to allow for a continuous flow of the solvent through the column.

HPLC separation of DS16E1. Two samples were prepared from the fractions containing antigenic activity recovered from the 250 ul C_{18} Sep Pak of DS16E1 for injection. The first, DS16E1-A, represented the first 50% MeOH elution of the C_{18} Sep Pak and contained 84% of the total antigenic activity recovered from the C_{18} Sep Pak. The solvent was removed in vacuo and the residue was taken up in 100 ul of the HPLC

Table 12. Recoveries from the C_{18} Sep Pak treatment of DS16E1.

Fraction	Quantity[a]	% Recovery[b]
	50 ul load	
0%	-0-	-0-
5%	-0-	-0-
1-50%	0.183±0.024	86
2-50%	0.013±0.001	6.1
100%	0.017±0.003	8.0
Recovered	0.212±0.028	
Pre-load	0.213±0.034	
	250 ul load	
0%	-0-	-0-
5%	-0-	-0-
1-50%	1.05±0.17	84
2-50%	0.13±0.01	10
100%	0.07±0.01	6.0
Recovered	1.25±0.19	
Pre-load	1.04±0.08	

[a]Antigenic activity recovered from DS16E1 as ug scopolamine eqs./g DWT.
[b]Antigenic activity recovered, expressed as a percent of total recovered.

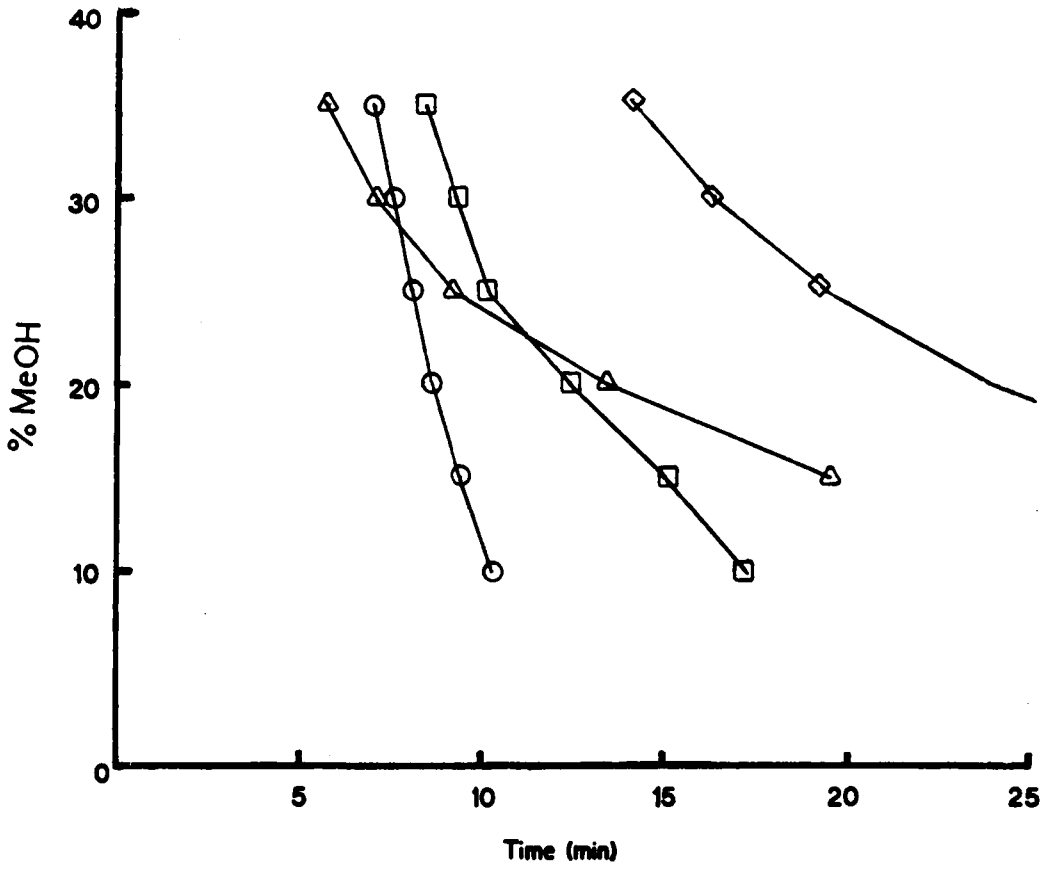


Figure 4. Retention time of standards as a function of methanol content of the mobile phase. Scopolamine (○), norscopolamine (△), 6-hydroxyhyoscyamine (□), and hyoscyamine (◇).

solvent. Half of this volume was taken and diluted with 50 ul of solvent. The second half was taken and diluted with 50 ul of solvent containing 50 ug scopolamine HBR, 50 ug hyoscyamine HBr, and 150 ug 6-hydroxyhyoscyamine.

A second sample, DS16El-B, was prepared from the second 50% MeOH elution which contained 10% of the antigenic activity. After removal of the C_{18} Sep Pak solvent the residue was taken up in 100 ul of HPLC solvent, but no portion was taken for internal standards. This sample was prepared to determine if the antigenic material in it was different from that contained in the first 50% MeOH elution.

Ten microliter injections were made of the DS16El-A preparations, and 1 ml fractions were collected for 40 minutes for later assay by RIA. The quantity of the internal standards/10 ul injection and the retention times of the external standards are shown in Table 13. The chromatograms for both injections are superimposed in Figure 5A for direct comparison of absorbance peaks.

No 229 nm absorbing peak attributable to scopolamine was detected in the chromatogram of DS16El-A. A new absorption peak appeared with DS16El-A containing internal scopolamine which coeluted with the external standard (10.5 min). A major absorption peak coeluted with external 6-hydroxyhyoscyamine (14.8 min). A small absorption peak coeluted with external hyoscyamine standard (32 min). The sizes of both of these peaks were increased with internal standards. Norscopolamine was not included with the set of internal standards, but its elution was separately determined at ca. 18 min. No absorption peak was seen that corresponded to norscopolamine.

Table 13. Alkaloid Standards in DS16E1-A and their retention times.

Standard	Content of internal standard[a]	Elution time (min) of external standard
Scopolamine	0.5 ug	10.5 min
6-Hydroxyhyoscy.	1.5 ug	14.8 min
Norscopolamine	---	18 min
Hyoscyamine	0.5 ug	32 min

[a]In 10 ul injection of DS16E1-A.

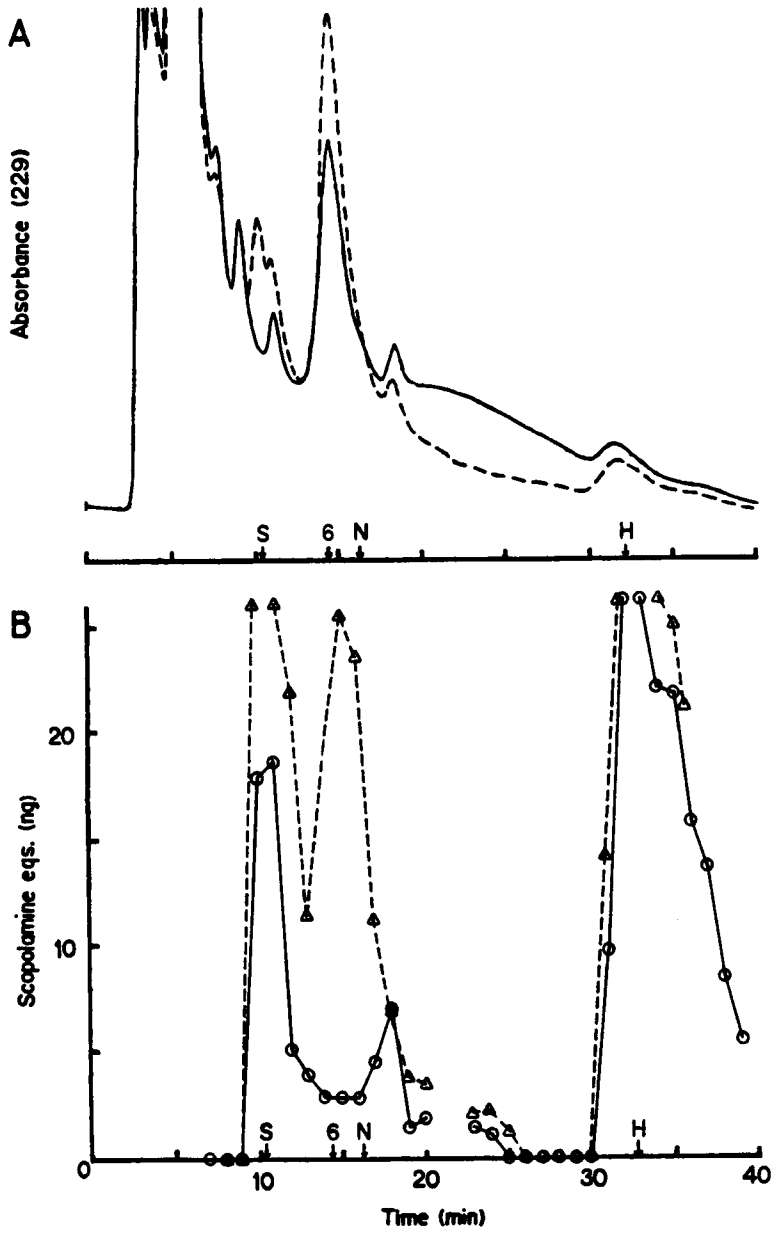


Figure 5. Analysis of DS16E1-A. A: Absorbance (229 nm) chromatogram of DS16E1-A (——) and DS16E1-A containing internal standards (----). B: RIA of the eluted fractions with antiserum HSA-12 of DS16E1-A (——) and DS16E1-A containing internal standards (---). Arrows on abscissa mark the elution times of separately injected external standards: scopolamine (S), 6-hydroxyhyoscyamine (6), norscopoolamine (N), and hyoscyamine (H).

Because 10% of the activity in DS16E1 was eluted in DS16E1-B, this second 50% MeOH fraction was examined to determine if this activity was due to qualitative differences in its antigenic composition compared to DS16E1-A. A different preparation of the mobile phase was used (pH 7.0), and this produced an earlier elution pattern for the sample and the standards. One ml fractions were again taken for later RIA analysis of separation of antigenic components.

About 85% of the entire sample DS16E1-B was injected and the chromatogram is shown in Figure 6. Inspection of the chromatogram reveals no detectable peak where authentic scopolamine eluted. A very small peak that coeluted with 6-hydroxyhyoscyamine standard is seen. A major peak coeluted with hyoscyamine standard.

Radioimmunoassay of the HPLC fractions. The fractions collected from DS16E1-A containing no internal standards was assayed in triplicate with both antisera. The graph of the HSA-12 RIA is shown in Figure 5B and for the KLH-10 RIA in Figure 7. DS16E1-A containing internal standards was assayed with antiserum HSA-12 only and this is also shown in Figure 5B. Antigen activity (as scopolamine equivalents) was plotted against elution times of the fractions; The detection limit of activity in the fractions collected was set at 1 ng/ml. The locations of the elution times of the external standards are indicated by arrows on the abscissa of the graphs.

The presence of five antigenic components in DS16E1-A were detected by both antisera, and the elution times of each were identical with both antisera. The first activity peak eluted at ca. 10.5 min.

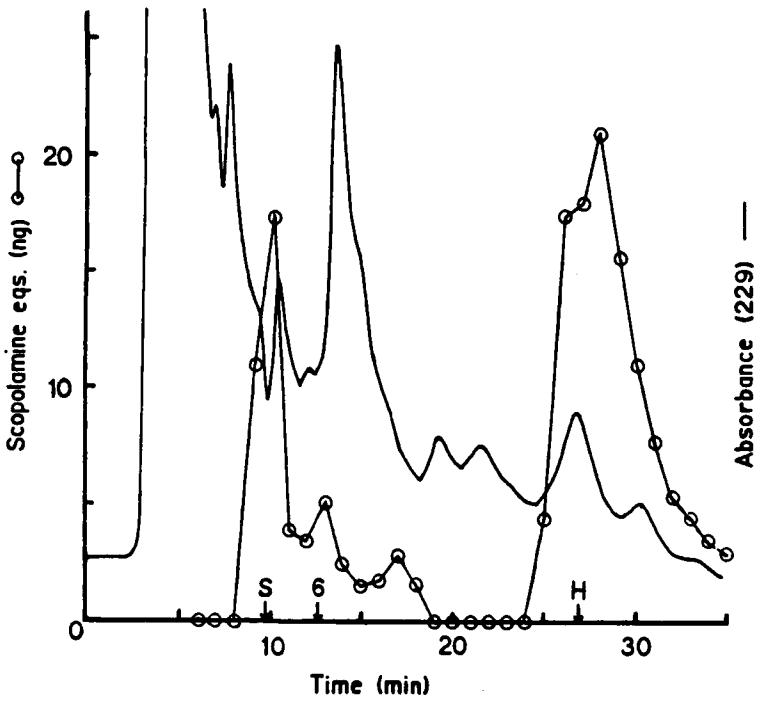


Figure 6. Analysis of DS16E1-B. Absorbance (229 nm) chromatogram and RIA of the eluted fractions with antiserum HSA-12. Arrows on abscissa mark the elution times of separately injected external standards.

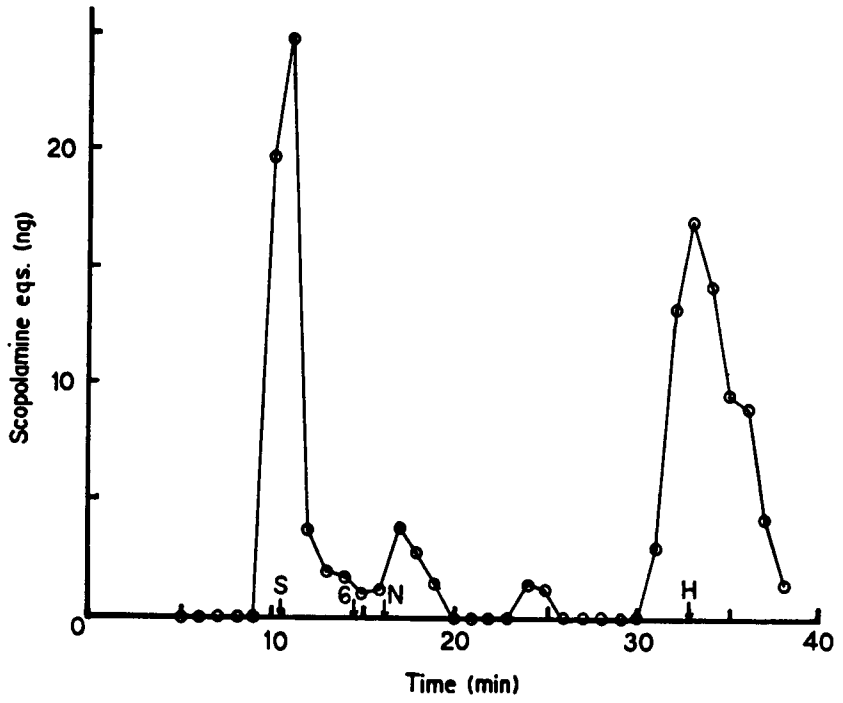


Figure 7. RIA of the eluted fractions of DS16E1-A with antiserum KLH-10. Arrows on abscissa mark the elutions times of separately injected external standards.

This peak corresponded to scopolamine; it coeluted with external scopolamine, and the size of the peak increased with internal scopolamine spiking of the sample. The quantity of scopolamine in the sample was below the sensitivity of the UV detector (no corresponding peak appeared in the chromatogram of this sample in Figure 5A), but the approximate 1000-fold increase in sensitivity for this compound by the RIA allowed the clear detection of it.

A second antigenic component eluted at ca. 14 min. The quantity of activity present was too small to be resolved as a distinct peak separate from the tails of the adjacent activity peaks. The activity present is above the detection limit, but the lack of its resolution is accounted for by the large sampling interval (fractions were collected at 1 min intervals). This component is tentatively identified as 6-hydroxyhyoscyamine. The elution time corresponded with internal and external 6-hydroxyhyoscyamine, and the size of the activity peak was increased with the internal spike of this antigen. The ratio of activity peaks of DS16E1-A to DS16E1-A containing internal standards is much greater then the ratio of their absorption peaks (compare peaks 14.5 min between panels A and B of Figure 5). This indicates the presence of a non-antigenic compound that absorbs at 229 nm and coelutes with 6-hydroxyhyoscyamine under the chromatographic conditions used.

A third peak of antigenic activity eluted at 18 min. This component eluted after norscopolamine and represents an unidentified antigenic component in the extract. A fourth activity peak also

representing a second unidentified antigen eluted at 23 min. Neither component exhibited a corresponding absorbance peak.

The fifth peak of antigenic activity detected by the RIA behaves as hyoscyamine. The activity peak coeluted with external hyoscyamine (ca. 32.5 min), and the peak size increased with the internal spike of the sample.

The fractions collected for DS16E1-B were assayed with the HSA-12 RIA. Figure 6 shows the elution and separation of four antigenic components. Three peaks eluted at the times expected for the authentic compounds scopolamine, 6-hydroxyhyoscyamine, and hyoscyamine. No additional activity peaks were resolved in this sample. The pH of the mobile phase for this sample was pH 7.0. This sufficiently changed the selectivity of the mobile phase to allow for the partial separation of 6-hydroxyhyoscyamine from the major non-antigenic absorbance peak it coeluted with in DS16E1-A.

The antigenic activity under each peak as measured by antiserum HSA-12 was totalled for both DS16E1-A and DS16E1-B. The percentage of the total antigenic activity that each component represents is shown in Table 14. These results indicated that other antigenic compounds (assumed to be tropane alkaloids) in addition to scopolamine are produced by D. stramonium in culture. Scopolamine accounted for only 20% of the activity measured in the extract with antiserum HSA-12. Hyoscyamine, the major product, accounted for 70% of the antigenic activity. The remaining 10% of the activity was due to other "novel" antigenic components in the extract. The profile of RIA activity in Table 14 shows DS16E1-B is nearly identical to DS16E1-A with

Table 14. Antigenic profile of DS16E1 after separation by HPLC.

	DS16E1-A		DS16E1-B	
Putative antigen	Activity[a]	% Recovery[c]	Activity[b]	% Recovery[c]
Scopolamine	46.5	19	33.7	20
6-Hydroxyhyoscy.	6.1	3	10.4	6
Unknown #1	14.4	6	6.7	4
Unknown #2	3.4	1	-	-
Hyoscyamine	>172	71	114.4	70

[a]Antigenic activity recovered as ng scopolamine egs. in a 10 ul injection of DS16E1-A as measured by the HSA-12 RIA.

[b]Antigenic activity recovered as ng scopolamine eqs. in a 85 ul injection of DS16E1-B as measured by the HSA-12 RIA.

[c]The percentage of the total activity recovered accounted for by each antigenic component.

scopolamine and hyoscyamine accounting for 90% of the total RIA activity. These results show the activity eluted in the second 50% MeOH wash of the 250 ul secondary Sep Pak treatment of DS16E1-0% was qualitatively the same as DS16E1-A and not due to the later elution of an additional compound with antigenic activity.

The relative contributions to the total antigenic activity by the five components in DS16E1-A when assayed with the more specific antiserum KLH-10 is shown in Table 15. Scopolamine was found to account for 38% of the antigen activity of the preparation. This was twice the activity represented by scopolamine with antiserum HSA-12, but hyoscyamine was still the major antigenic component of the extract as it accounted for 52% of the activity. The other active components resolved in the separation again accounted for 10% of cross-reactive components. These results indicated that even though antiserum KLH-10 has only a low cross-reactivity for hyoscyamine, the quantity of hyoscyamine accumulated is still too great for this antiserum to selectively measure scopolamine only in simple extract.

The yields of the alkaloids, as identified here, were calculated from antiserum cross-reactivity (Table 4, p. 35), and these estimates are given in Table 15. In the 250 ul volume of DS16E1, 13.5 ug of hyoscyamine were recovered, and this was ca. 25-times the quantity of scopolamine. The yields of alkaloids from callus tissue of D. stramonium line #16 were then calculated as 1.4 ug/g DWT, 4.7 ug/g DWT, and 35 ug/g DWT for scopolamine, 6-hydroxyhyoscyamine, and hyoscyamine respectively.

Table 15. RIA quantitation of antigenic components contained in DS16E1-A as determined by antiserum KLH-10.

Putative antigen	Activity[a]	% Recovery[b]	Quantity[c]
Scopolamine	103	38	0.52
6-hydroxyhyoscy.	5.8	2	1.8
Unknown #1	16.4	6	---
Unknown #2	5.2	2	---
Hyoscyamine	137	52	13.2

[a]Antigenic activity recovered as ng scopolamine equivalents in a 10 ul injection.

[b]The percentage of the total activity recovered accounted for by each antigenic component.

[c]Quantity of each antigenic component in ug/250 ul DS16E1. Calculated by: Scopolamine eqs. X 5 (dilution factor) X 100% divided by % cross-reactivity (Table 4, p.35) to convert to ug compound.

A sample of DS16E1 was sent to Drs. Y. Yamada and T. Hashimoto of Kyoto University, Japan, for GLC and GC-MS analysis of the tropane alkaloids (Yamada and Hashimoto, 1982). Hyoscyamine was separated and identified with both methods and was the major alkaloid component in the extract. A small peak at the location of scopolamine was seen in GLC but the quantity was at the limit of detection. GC-MS identification of this peak could not be made because of insufficient quantity of the compound in the sample. There was no indication of the presence of 6-hydroxyhoscyamine, but a small peak was detected by GLC whose retention time corresponded to littorine. There was no indication of the presence of any other tropane alkaloid in the extract.

A second preparation of DS16E1 was analyzed by HPLC. One half of the sample received a spike of internal standards (similar to DS16E1-A) which included littorine. The portion of the sample containing no internal standard was injected, and a small absorbance peak was identified whose retention time corresponded to that of separately injected littorine standard (data not shown). Furthermore, the size of the peak increased with the internal spiking of littorine standard. This peak eluted ca. 1.75 min before scopolamine. No corresponding antigenic activity peak was seen in the RIA using either of the antisera. These results indicate littorine was present in the extract, but its presence could not be detected by the RIA. The retention time determined for littorine also eliminates it as the possible identity of any of the unidentified antigenic components.

CHAPTER IV

CONCLUSIONS

An RIA was established here for use in investigations concerning the production of scopolamine by cultured cells of Datura. Scopolamine-binding antisera were produced using the immunogen described by Weiler et al. (1981) and a RIA procedure was set up that could be routinely performed with the facilities available to our laboratory. Several parameters of the KLH-10 RIA are compared with the RIA of Weiler et al. as shown in Table 16. The primary contrast between the RIAs is the demonstration here that the commercially prepared (N-methyl[^{3}H])-scopolamine methylchloride can be used as the labelled antigen in the RIA. The advantages of this are the labelled antigen did not have to be synthesized and the higher specific activity of the commercial compound allowed an approximate 8-fold increase in the sensitivity of the assay range (as judged from the mid-points of the scopolamine standard curves). However, Weiler et al. were able to obtain an antiserum with an approximately 6.5 lower cross-reactivity by hyoscyamine.

The ability of the RIA to measure scopolamine specifically in simple ethanolic extracts of tissue cultures needed to be verified because of the low level of cross-reactivity by hyoscyamine with antiserum KLH-10. When the alkaloids contained in an extract of Datura stramonium callus tissue were separated by HPLC chromatography, 4-5 peaks of antigenic activity were recognized using RIA. Scopolamine, 6-hydroxyhyoscyamine, and hyoscyamine were tentatively identified by

Table 16. Comparison of the KLH-10 RIA with the RIA described by Weiler et al. (1981).

Parameter	KLH-10	Weiler et al.
Labelled antigen	Scopolamine MeCl	Scopolamine
Specific activity	72 mCi/mMol	0.67 mCi/mMol
Useable range of standard curve	0.15-3.0 ng	0.5-50 ng
Hyoscyamine cross-reactivity	5%	0.8%
Antiserum titre	1/250	1/125
Protein conjugate (immunogen)	Key-hole limpet haemocyanin	Human serum albumin
Application	Tissue cultures	Plant tissues

this combination of HPLC and RIA. Littorine was also tentatively identified as a non-antigenic component. The scopolamine peak was found to account for less than half of the antigenic activity. The presence of hyoscyamine and the determination that it was the major antigenic component accumulated was established unequivically by GC-MS. Thus it is concluded that the RIA using antiserum KLH-10 cannot be directly applied to the quantitation of scopolamine in simple extracts of callus tissue because of the excessive quantities of hyoscyamine present. With these extracts the cross-reactivity of the antiserum described by Weiler et al. would overestimate the amount of the amount of scopolamine in the extracts by 36% due to the amount of hyoscyamine and 6-hydroxyhyoscyamine present.

The results of the analysis of D. stramonium can be generalized and extended to the application of the RIA for the determination of scopolamine in tissue cultures of other species which have the biosynthetic capacity to produce this compound. As was shown here, the quantities of cross-reacting compounds produced in a given culture line must be determined to ensure that it is scopolamine which is specifically being measured and not compounds with low cross-reactivities that are accumulated in quantities which overwhelm the specificity of the RIA. The report by Oksman-Caldentey and Strauss (1986) on the variation of scopolamine content in protoplast-derived cell culture clones of Hyoscyamus muticus was based on the antisera of Weiler et al. (1981). They demonstrated significant variation in yields was occurring between clonal cultures, but the nature of the

antigenic activity was not determined. Their conclusion attributing the variation in their cultures to changes in scopolamine production is precarious because they provide no verification that it was actually scopolamine which was measured in the clones and not the summation of activity from other antigenic components which may have been accumulated in significantly greater amounts.

When cross-reacting compounds are determined to be produced in tissue cultures in significant quantities, a separation step must be introduced to remove or at least minimize the effects of these compounds before specific quantitation of scopolamine can be made by the KLH-10 RIA described here. This separation may potentially be achieved by solid-phase extraction (e.g. Sep Paks) or by coupling the RIA to HPLC. However, the inability to directly assay simple extracts will increase the material and labor costs per sample and decrease the number of samples analyzed per unit of time. The RIA may be applied to simple extracts if the focus of the quantitation is expanded to include the metabolic precursors to scopolamine, 6-hydroxyhyoscyamine and hyoscyamine, to which an antiserum is cross-reactive. For the purpose of examining the biochemical basis of variation in yields of secondary metabolites of Datura, then, antiserum HSA-12 could be used to measure the total products in the scopolamine-type branch of tropane alkaloid metabolism.

The specific quantitation of scopolamine in unpurified extracts of Datura tissue cultures requires an antiserum be obtained that has a much lower cross-reactivity for the closely related tropane alkaloid metabolites. The antisera described here and by Weiler et al. (1981)

are sensitive to these compounds because they are accumulated in tissue cultures in substantially greater quantities than scopolamine. One approach for obtaining a more specific antiserum is to use a different scopolamine immunogen for immunizing rabbits. Such an immunogen would have scopolamine conjugated to the carrier protein via the tropic acid moiety. This would increase the exposure of the epoxide and N-methyl structures during antibody elicitation in rabbits and thereby increase the likelyhood for obtaining antisera with much greater specificities for these structures. A larger group of immunized rabbits would also provide a larger pool of antisera from which to screen for a specific antiserum.

An alternative method for obtaining highly specific antisera would be the use of monoclonal antibodies. Once established, a monoclonal antibody system can essentially provide a limitless supply of antisera that is uniquely specific for scopolamine. However, a monoclonal antibody system is technically much more demanding to develop than a polyclonal system. The expense to set up a monoclonal antibody system is also much greater, but the initial high development costs may be offset in the longrun by the reduced cost per sample processed.

The application of the RIA to simple extracts of the stock cultures demonstrated that all five Datura species expressed a biosynthetic capacity to produce antigenic compounds. The coupled HPLC/RIA methodology used here to identify the major antigenic compounds in the D. stramonium line can be used to help identify the antigenic compounds produced by the other four species.

Variation in the yields of antigenic compounds produced by the callus cultures was observed with the RIA. Reassaying of the cultures over time indicated there were fluctuations in the antigen (alkaloid) yields of the callus. Variation in yields was also observed between species and between lines established from individual plants for two species. Cloning of cultures is needed eliminate any residual variation originating from the initial explant. This can allow characterization of the cellular variation that arises during in vitro growth.

Alkaloid yields of Datura may be increased by the optimization of the basal medium and the supplementation with growth regulators. B5 medium was arbitrarily chosen as the basal medium for these cultures, but other formulations have been used. Medium optimization would be approached by assaying yields in cultures grown on different basal media and then selecting one which gives the best response for further optimization of the individual components of the medium. Each species would be examined individually since each may respond differently to different culture conditions. Growth regulator supplementation was shown to effect Datura culture yields. These effects could be optimized by examining different types of growth regulators over a range of concentrations. This optimization would primarily focus on the auxin source since auxin is essential for good growth but specific types may be inhibitory to alkaloid production. The (HSA-12) RIA would be very convenient for primary screening of the culture yields during these studies. The HPLC methodology could then be used to determine the specific composition of the antigenic reactants.

LIST OF REFERENCES

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Balandrin, M.F., J.A. Klocke, E.S. Wurtele, and W.H. Bollinger. 1985. Natural plant chemical: Sources of industrial and medicinal materials. Science 228:1154-1160.

Chan, W-N., and E.J. Staba. 1965. Alkaloid production by Datura callus and suspension tissue cultures. Lloydia 28:55-61.

Chaprin, N. and B.E. Ellis. 1984. Microspectrophotometric evaluation of rosmarinic acid accumulation in single plant cells. Can. J. Bot. 62: 2279-2282

Constabel, F., S. Rambold, K.B. Chatson, W.G.M. Kurz, and J.P. Kutney. 1981. Alkaloid production in Catharantus roseus (L.) G. Don. VI. Variation in alkaloid spectra of cell lines derived from one single leaf. Plant Cell Reports 1:3-5.

Cordell, G.A. 1981. Introduction to Alkaloids: A Biogenic Approach. John Wiley and Sons, Inc., New York.

Curtin, M.E. 1983. Harvesting profitable products from plant tissue culture. Biotechnology 1:649-657.

Desbuquois B., and G.D. Aurbach. 1971. Use of polyethylene glycol to separate free and antibody-bound peptide hormones in radioimmunoassays. J. Clin. Endocr. 33:732-738.

Deus-Neumann, B., and M.H. Zenk. 1982. Exploitation of plant cells for the production of natural compounds. Biotech. Bioeng. 24: 1965-1974.

Deuz, P., S. Chamart, M. Hanocq, and L. Molle. 1985. Comparison between thin-layer chromatography-densitometry and high performance liquid chromatography for the determination of hyoscyamine and hyoscyne in leaves, fruit and seeds of Datura (Datura spp.). J. Chrom. 329:415-421.

Dougall, D.K. 1979. Production of biologicals by plant cell cultures. pp. 53-70. IN: Cell Substrates. J.C. Petricciani, H.E. Hopps, and P.J. Chappie eds. Plenum Publishing Corp., New York.

Dougall, D.K. 1985. Chemicals from plant cell cultures: Yields and variation. pp. 179-190. IN: Biotechnology in Plant Science. Academic Press, New York.

Dougall, D.K., J.M. Johnson, and G.H. Whitten. 1980. A clonal analysis of anthocyanin accumulation by cell cultures of wild carrot. Planta 149:292-297.

Evans, W.C. 1979. Tropane alkaloids of the Solanaceae. pp. 241-254. IN: The Biology and Taxonomy of the Solanaceae. R.N. Lester, and A.D. Skelding, eds. Academic Press, London.

Fujita, Y., and M. Tabata. 1987. Secondary metabolites from plants--pharmaceutical applications and progress in commercial production. pp. 169-185 IN: Plant Tissue and Cell Culture. C.E. Green, D.A. Somers, W.P. Hackett, and D.D Biesboer, eds. Alan R. Liss, Inc., New York.

Fowler, M.W. 1987. Process systems and approaches for large scale plant cell culture. pp. 459-471. IN: Plant Tissue and Cell Culture. C.E. Green, D.A. Somers, W.P. Hackett, and D.D. Biesboer, eds. Alan R. Liss, Inc., New York.

Gamborg, O.L. 1970. The effects of amino acids and ammonium on the growth of plant cells in suspension culture. Pl. Phys. 45:371-375.

Griffin, W.J. 1976. Agronomic evaluation of Datura candida--A new source of hyoscine. Economic Botany 30:361-369.

Hashimoto, T., and Y. Yamada. 1986. Hyoscyamine 6-beta-hydroxylase, a 2-oxoglutarate-dependent dioxygenase, in alkaloid-producing root cultures. Plant Physiol. 81:619-625.

Hiraoka, N., and M. Tabata. 1974. Alkaloid production by plants regenerated from cultured cells of Datura innoxia. Phytochem. 13:1671-1675.

Krikorian, A.D., and F.C. Steward. 1969. Biochemical differentiation: The biosynthetic potentialities of growing and quiescent tissue. pp. 226-326 IN: Plant Physiology. F.C. Steward, ed. Vol. 5B. Academic Press, New York.

Landon, J., and A.C. Moffat. 1976. The radioimmunoassay of drugs. The Analyst 101:225-243.

Leete, E. 1979. Biosynthesis and metabolism of the tropane alkaloids. Planta Med. 36:97-112.

Luckner, M., and L. Nover. 1977. I. Expression of Secondary Metabolism: An Aspect of Cell Specialization of Microorganisms, Higher Plants, and Animals. pp. 1-101. IN Secondary Metabolism & Cell Differentiation. Springer-Verlag, Berlin.

Meins, F.Jr. 1983. Heritable variation in plant cell culture. Ann. Rev. Pl. Phys. 34:327-346.

Mizukami, H., M. Konoshima, and M. Tabata. 1978. Variation in pigment production in Lithospermum erythrorhizon callus cultures. Phytochem. 17:95-97.

Netien, M.A., and J. Combet. 1966. Obtension de tissus de tige de Datura metal (L.) cultives in vitro: modifications biochimiques azotees. Compt. Rend. 262D:1084-1086.

Ogino, T., N. Hiraoka, and M. Tabata. 1978. Selection of high nicotine producing cell lines of tobacco callus by single-cell cloning. Phytochem. 17:1907-1910.

Oksman-Caldentey, K.-M., and A. Strauss. 1986. Somaclonal variation of scopolamine content in protoplast-derived cell culture clones of Hyoscyamus muticus. Planta Medica 52:6-12.

Phillipson, J.D. 1979. The search for new drugs from plants. Pharm. J. 222:310-312.

Plank, K.-H., and K.G. Wagner. 1986. Determination of hyoscyamine and scopolamine in Datura innoxia plants by High Performance Liquid Chromatography. Z. Naturforsch. 41c:391-395.

Schulte, U., H. El-Shagi, and M.H. Zenk. 1984. Optimization of 19 Rubiaceae species in cell culture for the production of antraquinones. Plant Cell Rep. 3:51-54.

Staba, E.J. 1969. Plant tissue culture as a technique for the phytochemist. pp. 76-106. IN: Recent Advances in Phytochemistry, Vol. 2. Appleton-Century-Crofts, New York.

Staba, E.J. 1985. Milestones in plant tissue culture system for the production of Secondary products. J. Nat. Prod. 48:203-209.

Tabata, M. 1977. Recent advances in the production of medicinal substances by plant cell cultures. pp. 3-16. IN: Plant Tissue Culture and Its Biotechnological Application. W. Barz, E. Reinhard, and M.H. Zenk, eds. Springer-Verlag, Berlin.

Tabata, M., and N. Hiraoka. 1976. Variation of alkaloid production in Nicotiana rustica callus cultures. Physiol. Pl. 38:19-23.

Virtanen, R., J. Kanto, and E. Iisalo. 1980. Radioimmunoassay for atropine and l-hyoscyamine. Acta Pharmacol. et Toxicol. 47:208-212.

Weiler, E.W. 1977. Radioimmuno-screening methods for secondary plant products. pp. 266-277. IN: Plant Tissue Culture and Its Biotechnological Application. W. Barz, E. Reinhard, and M.H. Zenk, eds. Springer-Verlag, Berlin.

Weiler, E.W., J. Stockigt, and M.H. Zenk. 1981. Radioimmuno-assay for the quantitative determination of scopolamine. Phytochem. 20:2009-2016.

Weiler, E.W., and M.H. Zenk. 1976. Radioimmunoassay for the determination of digoxin and related compounds in Digitalis lanata. Phytochem. 15:1537-1545.

Yamada, Y. and T. Hashimoto. 1982. Production of tropane alkaloids in cultured cells of Hyoscyamus niger. Plant Cell Rep. 1:101-103.

Yamada, Y., and T. Hashimoto. 1984. Secondary products in tissue culture. pp.561-604. IN: Application of Genetic Engineering to Crop Improvement. G.B. Collins and J.G. Petolino, eds. Martinus Nijhoff/Dr. W. Junk, Dordrecht.

Zenk, M.H. 1978. The impact of plant cell culture on industry. pp. 1-14. IN: Frontiers of Plant Tissue Culture 1978. T.A. Thorpe, ed. IAPTC, Calgary.

Zenk, M.H., H. El-Shagi, H. Arens, J. Stockigt, and B. Deus. 1977. Formation of the indole alkaloids serpentine and ajmalicine in cell suspension cultures of Catharanthus roseus. pp. 27-43. IN: Plant Tissue Culture and Its Biotechnological Application. W. Barz, E. Reinhard, and M.H. Zenk, eds. Springer-Verlag, Berlin.

Zenk, M.H., H. El-Shagi, and U. Schulte. 1975. Anthraquinone production by cell suspension cultures of Morinda citrifolia. Planta Medica, Suppl. 79-101.

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