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A STEROL-BINDING ASSAY FOR POTATO GLYCOALKALOIDS

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Abstract—A method is described for quantitative analysis of potato glycoalkaloids based on their ability to complex with free sterols in alcoholic solution, the amount of unbound sterol being inversely proportional to the amount of alkaloid present. Since the alkaloid–sterol complex is soluble, unbound sterol is estimated by GLC.

INTRODUCTION

Since their discovery in potato in 1826 [1], steroidal glycoalkaloids have been quantified by a number of methods. The earliest methods were gravimetric, but in 1937 Conner [2] reported a volumetric method based on estimation of sugars liberated by acid hydrolysis. However, Alberti's use of Marquis reagent (conc. H_2SO_4 + formaldehyde) for colorimetric determination of 'solanine' [3] became the most widely-adopted method and was continually improved over a number of years [4, 5]. Later work showed that chromogens could also be produced by treating with H_3PO_4 and *para*-formaldehyde [6], or with conc. HCl and antimony trichloride [7], and Cadle *et al.* [8] have linked the latter method with densitometry on TLC plates. Although sometimes very sensitive, the chromogen methods have the drawback of using toxic and/or corrosive chemicals and being highly dependent on the purity of the extracted alkaloids. Fitzpatrick and Osman [9] circumvented some of these problems by devising a non-aqueous titration of the aglycone nitrogen group with bromophenol blue. More recently, GLC methods have been described by Herb *et al.* [10] who separated and quantified permethylated derivatives, and by Roosen-Runge and Schneider [11] who used silylated derivatives. Unfortunately, the latter authors gave no details of peak characteristics, quantitative data or recovery values. Steroidal alkaloids (including the aglycone of the potato alkaloids, solanidine) have been separated by HPLC [12] and quantitative analyses have recently been reported using this technique [17].

This paper describes an assay of potato glycoalkaloids (PGAs) based on their ability to complex with free sterols *in vitro*, the amount of unbound sterol being inversely proportional to the amount of alkaloid. The method is a modification of the successful radioligand assay of tomatine devised by Heftmann and Schwimmer [13]. However, because PGAs form complexes with sterols which are soluble in alcoholic solution (unlike tomatine–sterol complexes) and thus cannot be separated by centrifugation, the amount of unbound sterol in solution cannot be quantified by using labelled sterol and counting radioactivity, but instead is determined by GLC.

RESULTS AND DISCUSSION

The radioligand assay of tomatine [13] employs [4-^{14}C]-cholesterol, but previously it was shown that (a) PGA binds sterols less readily than tomatine and (b) cholesterol was the least readily bound of the sterols tested [14]. For these studies stigmasterol was chosen, not only because of its greater complexing ability with PGA, but also because it is readily available in pure form at low cost compared with campesterol which is expensive and sitosterol which is usually impure.

In the tomatine assay, Heftmann and Schwimmer [13] recommended heating the reaction mixture for a few minutes followed by cooling in a fridge overnight. The latter step, which presumably aids precipitation of the complex, is of no importance in this PGA assay since PGA–sterol complexes are soluble in ethanol. Since the necessity for heating in the tomatine assay has already been questioned [14], preliminary experiments were carried out to decide its importance in a sterol-binding PGA assay.

PGA (0.5 mg/ml ethanol) was reacted with an ethanolic stigmasterol solution for 5 min either at 90° or at room temperature. Tubes were then left at room temperature for varying periods up to 16 hr to decide if a longer incubation time would be required to compensate for lower incubation temperature. Zero time controls and 16 hr controls showed that there was no reduction in sterol with time, or as a result of the 5 min heating, all values for residual stigmasterol in the EtOAc extract being in the range 0.60–0.62 μg/μl. Tubes containing PGA had less free sterol than controls but, whether heated or not and irrespective of sampling time between 5 min and 16 hr concentrations were in the range 0.34–0.42 μg/μl. Heating, therefore, did not appear to be necessary for, or to accelerate, complex formation, and at room temperature maximum binding was observable after 5 min. Segal *et al.* [15] found that binding of tomatine with sterols occurred too rapidly to measure and it is very

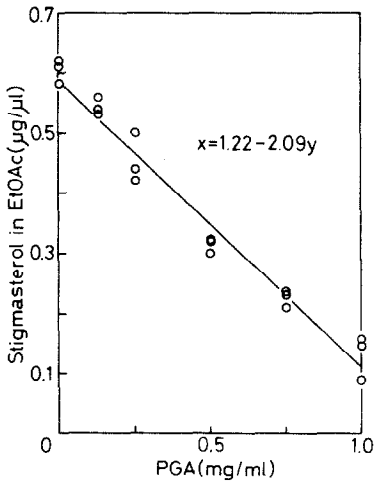

Fig. 1. Calibration graph of concentration of stigmasterol in the EtOAc extract vs concentration of PGA. Points represent three replicate determinations of unbound sterol at different PGA concentrations. The line of fit was calculated by regression analysis. (Correlation coefficient $r = -0.99$.)

likely that, *in vitro*, complex formation between PGA and sterol is complete in less than 5 min. However, to allow an ample margin for error an incubation time of 1 hr (at room temperature) was employed in all subsequent experiments.

A calibration graph prepared under these conditions is shown in Fig. 1. A strong negative correlation ($r = -0.99$) exists between the concentration of unbound stigmasterol and the concentration of PGA up to 1 mg/ml alkaloid. Above this concentration, PGA did not remain in solution. The binding data from which the graph was constructed agreed closely with those previously reported [14].

Experiments with pure α-solanine showed that its sterol-complexing properties were identical with those of PGA. α-Chaconine was not available for testing but since PGA is a mixture of these two alkaloids it follows that α-solanine and α-chaconine must possess very similar sterol-complexing properties. In contrast, the aglycone of these glycosides, solanidine, did not show any evidence of complexing with stigmasterol, a finding which is in keeping with the apparent inability of the aglycone of α-tomatine, tomatidine, to bind with sterols in organic solution [14, 16]. The sugar moiety thus appears to be a prerequisite for sterol-binding by PGA, at least *in vitro*.

The efficiency of the overall extraction and assay procedure was determined by measuring recovery of authentic PGA added to extracts of tuber peel, the endogenous PGA of which was determined separately. The recovery of added alkaloid in each of three replicate runs was in excess of 90% (Table 1). It is, of course, probable that this value would vary with the extraction procedure (many of which exist) but no attempt has been made here to compare these. Nor is the method which has been used claimed to be in any way superior to others.

This assay method avoids a number of the disadvantages associated with some of the other methods described above. For example, it does not employ expensive, corrosive or highly toxic chemicals and it

Table 1. Efficiency of the extraction and assay procedure

No.	PGA added (mg)	Total PGA found (mg)	Added PGA recovered (mg)	% Recovery
1*a*	0	9.0	—	—
1*b*	17.4	26.2	17.2	99.4
2*a*	0	11.5	—	—
2*b*	17.4	27.5	16.0	92.0
3*a*	0	6.3	—	—
3*b*	17.4	22.5	16.2	93.1
			Mean ± s.e.	94.8 ± 1.88

Three 100 g batches of potato peel were extracted and extracts split into two equal volumes, *a* and *b*. Authentic PGA in MeOH was added to flasks labelled *b* and MeOH only to controls labelled *a*. The amount of PGA added was checked using the GLC assay.

avoids the hazards and uncertainties of derivative formation. It measures intact glycosides, not the aglycone, and therefore does not require a hydrolysis step. Because of the specificity of sterol binding, the alkaloid need not be in as high a state of purity as is required for many of the colorimetric methods, and the strictly-standardized procedures commonly associated with such methods are not necessary here. The method is relatively rapid taking a maximum of only 1.5 hr although this could possibly be reduced by as much as 70% by reducing incubation time. Some weaknesses of this method are that it does not distinguish between the two constituent alkaloids (although prior separation by TLC could remedy this) and it is not quite as sensitive as direct GLC methods or some of the colorimetric methods.

EXPERIMENTAL

The PGA used was a mixture of α-solanine and α-chaconine extracted from etiolated potato sprouts. Methods for extraction, purification and confirmation of identity have been described previously [14]. Authentic α-solanine was purchased from Sigma Chemical Company, Poole, Dorset and authentic solanidine from Koch-Light Laboratories, Colnbrook, Bucks.

Extraction and estimation of endogenous PGA were conducted as follows. Potato tubers (cv Désirée) obtained from a local store were washed, surface-dried with tissue and peeled to a depth of 2 mm. Peel was immediately homogenized with 94% MeOH acidified with 2% HOAc (4 ml/g fr. wt.) and left for 5 hr with occasional stirring after which it was Buchner-filtered through Whatman No. 1 paper and re-extracted for 15 min using 2 ml extractant/g fr. wt. After filtration, extracts were bulked and reduced to dryness *in vacuo* at 45°. Flask contents were taken up in warm 2% HOAc and transferred to a 50 ml polypropylene centrifuge tube. Conc. NH_3 was added to pH 10.0. Tubes were heated in a H_2O bath at 80° for 30 min and then cooled at 4° overnight. Contents were centrifuged at 27 000 × *g* for 30 min at 4°, supernatants discarded and the pellet washed with 1% NH_3 and centrifuged as before. The pellet was dried in a $CaCl_2$ desiccator and extracted with MeOH in a 70° H_2O bath for 2 hr. After centrifuging as above, the supernatant was decanted, the pellet re-extracted as before, but for 1 hr, and the tubes again

centrifuged. Supernatants were bulked, reduced to dryness *in vacuo* at 35°, taken up in hot EtOH and made to vol. (10 or 25 ml) in 96% EtOH.

The reaction mixture was prepared by mixing 2 ml of extract or authentic ethanolic alkaloid soln with 0.5 ml of stigmasterol soln (containing 1.5 mg/ml stigmasterol and 0.1 mg/ml butylated hydroxytoluene in 96% EtOH). The purity of the stigmasterol was checked by GLC. After 1 hr at room temp. a 0.6 ml aliquot was removed, evapd to dryness in an air stream and taken up in 0.25 ml of a soln of 5α-cholestane in EtOAc (0.4 mg/ml). Taking up in EtOAc proved an important step as it readily dissolved unbound sterol but did not remove any PGA which could have caused problems in the GLC. The absence of PGA in this EtOAc extract was confirmed by TLC on Si gel G (0.25 mm) with 95% EtOH as solvent and modified Dragendorff's reagent as locating reagent. A 1 μl aliquot of this soln was injected onto a glass column (2 m × 6 mm) packed with 3% OV-101 on Gas Chrom Q. N_2 flow rate was 50 ml/min, column temp. isothermal at 250° and FID temp. 325°. 5α-Cholestane acted as internal standard and sterol was quantified using a pre-programmed Supergrator Mk II integrator. GLC traces showed only two peaks, cholestane at *ca* 5.3 min and stigmasterol at *ca* 11.5 min.

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