Fluorescence Polarization Immunoassays for Potato Glycoalkaloids

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Fluorescence polarization immunoassays (FPIAs) were developed for potato glycoalkaloids (GAs) using a polyclonal antiserum and a monoclonal antibody (MAb). Fluorescently labeled solanidine (AMF-SOL) was synthesized by coupling 4′-(aminomethyl)fluorescein (4′-AMF) to the hemisuccinate derivative of solanidine using an active ester method. Both polyclonal antibody (PAb) and MAb could quantify the major potato GAs in the 20−100 nM range; however, the affinities of PAb and MAb differed slightly among the individual GAs. PAb displayed the greatest affinity for α-chaconine, while MAb was more sensitive to changes in α-solanine levels. Affinity constants (K_{aff}) of PAb and MAb for AMF-SOL were estimated at 4.2×10^8 and 4.7×10^7 M^{-1}, respectively. Potato samples containing low, medium, and high levels of GAs were successfully analyzed by the PAb FPIA. Equilibrium in the FPIA reaction was reached within 1−2 min, and standard curves were stable for at least 14 days.

Keywords: *Monoclonal; polyclonal; antibody; solanidine; alkaloids; analysis*

INTRODUCTION

Glycoalkaloids (GAs) are naturally occurring toxic components of potatoes which are believed to confer resistance to Colorado potato beetle and potato leafhopper to the plant (Flanders et al., 1992; Gaffield et al., 1991; Roddick et al., 1988) but which can also cause human and livestock illness and even death (Baker et al., 1991; Morris and Lee, 1984). Wild potato varieties often contain high levels of GAs and are therefore attractive to plant breeders attempting to increase the hardiness of offspring. Unfortunately, the tubers of such offspring can contain greater than 20 mg of total GA/100 g of tuber, which is the generally accepted cutoff level between safe and unsafe potatoes (Slalina, 1990; Van Gelder, 1991).

Immunological methods for the analysis of GAs can eliminate the extensive extraction procedures and sample cleanup usually required for chromatographic analysis and avoid the use of toxic reagents often employed in the colorimetric analysis of GAs. Solid-phase immunoassays (IAs) such as enzyme immunoassay and radio immunoassay have been developed for the analysis of potato GAs (Morgan et al., 1983, 1985; Plhak and Sporns, 1992, 1994; Vallejo and Ercegovich, 1979). Such methods, while specific for GAs, generally display poor reproducibility and are therefore more suited to the generation of qualitative rather than quantitative results.

Fluorescence polarization immunoassays (FPIAs) are homogeneous assays that rely on the increase in the polarization of fluorescence when a fluorescently labeled hapten is bound to antibody (Ab) as compared to the polarization of fluorescence of the free labeled compound in solution (Colbert et al., 1991). Despite the higher limits of detection of homogeneous methods (including FPIA), relative to those attainable using solid-phase assays, homogeneous assays permit rapid analysis since the equilibrium between antigen and Ab is reached within minutes (Colbert et al., 1986). Since the FPIA reaction occurs entirely in solution, kinetics modeling of the Ab binding reaction is facilitated. Perhaps most importantly, results obtained using FPIA are highly reproducible, and standard curves remain stable for extended periods of time (Thomas et al., 1991; Zaninotto et al., 1992).

GAs are always present in potato tubers, often at relatively high levels. Therefore, the objective of this work was to determine the feasibility of using an FPIA to detect and quantify the major GAs likely to be present in cultivated potatoes. The successful development of such a procedure might allow for the screening of many potato samples during the early stages of breeding programs.

MATERIALS AND REAGENTS

Instrumentation. A Perkin-Elmer LS-50 luminescence spectrometer equipped with a pulsed xenon lamp source and an automatic interchangeable film polarizer wheel was used. The instrument was configured in the L-format, in which emission of fluorescence is detected at 90° to the direction of excitation.

The fluorescence polarization (FP) was determined in solutions contained in Spectrosil quartz cuvettes (Starna Cells, Inc., Atascadero, CA). Data were collected using a 486DX-33 cpu computer and processed by FLDM software (Perkin-Elmer). Competitive inhibition curves were generated using SOFTmax (Menlo Park, CA) and the equation

$$y = \frac{a - d}{1 + (x/c)^b} + d$$

where x = concentration of analyte, y = response (i.e., mP units), a = asymptote at low values of the x-axis, d = asymptote at high values of the x-axis, $c = x$ value corresponding to the midpoint between a and d values, and b = rate of transition between a and d values.

Materials. 4′-(Aminomethyl)fluorescein (4′-AMF) was purchased from Molecular Probes, Inc. (Eugene, OR). α-Chaconine, α-solanine, α-tomatine, solanidine, 4-(dimethylamino)-pyridine, and Tween 20 were obtained from Sigma Chemical Co. (St. Louis, MO). Pyridine was purchased from Fisher Scientific (Edmonton, AB). Succinic anhydride was obtained from BDH Inc. (Edmonton, AB). *N*-Hydroxysuccinimide, 1,3-dicyclohexylcarbodiimide, and *N*,*N*-dimethylformamide were obtained from Aldrich Chemical Co. (Milwaukee, WI).

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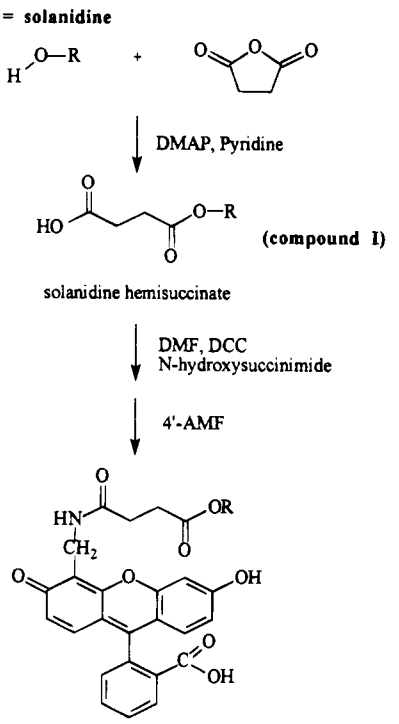

Figure 1. Reaction scheme for the synthesis of AMF-SOL.

Potato samples a, e, and f were as described by Plhak and Sporns (1992).

Polyclonal antiserum (PAb) was obtained from Plhak and Sporns (1992), and monoclonal antibody (MAb) was obtained from Plhak and Sporns (1994). Protein concentration, as IgG, was determined by measuring absorbance values at 280 (A_{280}) and 350 nm (A_{350}) against a PBS blank and using the following equation adapted from Harlow and Lane (1988):

$$[\text{protein}] = \frac{A_{280} - A_{350}}{1.35}\ (\text{mg/mL})$$

Stock solutions of GAs (α-solanine, α-chaconine, and α-tomatine) and solanidine were prepared in methanol to concentrations of 100 nM and stored at −20 °C.

PROCEDURES

Synthesis of Fluorescently Labeled Solanidine (AMF-SOL) (Figure 1). Solanidine hemisuccinate was synthesized according to the method of Plhak and Sporns (1992) and coupled to 4′-(aminomethyl)fluorescein dihydrochloride (4′-AMF) using the active ester method of Shipchandler et al. (1987). Solanidine hemisuccinate (21.1 mg) was reacted with 4.18 mg of *N*-hydroxysuccinimide and 6.24 mg of 1,3-dicyclohexylcarbodiimide in 625 μL of dry *N*,*N*-dimethylformamide (DMF) at room temperature for 30 h. Urea formed during the reaction was removed by filtration. To the active ester was added 12.42 mg of 4′-AMF, 12.5 μL of triethylamine, and 207.8 μL of dry DMF. The reaction mixture was stirred at room temperature for 20 h, and the DMF was removed under reduced pressure. The fluorescein-labeled solanidine was purified by normal-phase preparative thin-layer chromatography (TLC) on silica gel Kieselgel $60F_{254}$ 20 × 20 cm, 1000 μm thick plates (E. Merck, Darmstadt, Germany) using a solvent system of ethyl acetate/methanol/1% aqueous ammonia (80:20:1 v/v/v) (Plhak and Sporns, 1992). The band corresponding to fluorescently labeled solanidine (AMF-SOL) was scraped from the plate, the AMF-SOL dissolved in methylene chloride, and the solid-phase silica removed by filtration. The methylene chloride was removed under reduced pressure, and the residue was dried under vacuum over phosphorus pentoxide. The orange product (15.3 mg, 18.2 mmol) obtained was stored at −20 °C. A stock solution of AMF-SOL was prepared in methanol (4140 nM) and stored at 4 °C in the dark.

Standard FPIA Procedure. Equal volumes (usually 0.5 mL) each of 180 nM AMF-SOL in PBST, diluted MAb or PAb, and diluted glycoalkaloid or alkaloid standards were mixed in a test tube. Equilibration proceeded for 30 min at room temperature. FP readings were measured using an excitation wavelength of 496 nm (2.5 nm slit width) and an emission wavelength of 518 nm (7.5 nm slit width).

Antibody Dilution Curves. PAb was diluted (v/v) to cover the range between 1:50 and 1:4000. To 1.0 mL aliquots of diluted PAb were added 1.0 mL aliquots of AMF-SOL diluted in PBST for a final concentration in the FPIA reaction of 57.5 nM. FP measurements of equilibrated samples were made using an excitation wavelength of 496 nm (2.5 nm slit width), an emission wavelength of 518 nm (10.0 nm slit width), and an integration time of 10 s.

A MAb dilution curve was derived as above using doubling dilutions of MAb in PBST adjusted to pH 6.5 to give final concentrations from 0.62 to 0.002 mM in the FPIA reaction. AMF-SOL concentration was 60 nM in the final FPIA reaction. After equilibration, FP readings were taken as indicated for the standard protocol.

Standard Curves for Alkaloids and Glycoalkaloids. PAb (0.5 mL) diluted in PBST to give a final dilution of 1:200 was incubated with 0.5 mL of 60 nM AMF-SOL in PBST and 0.5 mL of each of the standards (α-chaconine, α-solanine, α-tomatine, and solanidine) diluted in PBST to give final concentrations in the FPIA reaction ranging from 5 to 5000 nM. FP values in the absence of analyte were obtained as above, except PBST substituted for analyte. The stability of the standard curve for α-chaconine was determined by assaying the same standards for a period of 23 days.

Stability of AMF-SOL in PBST. AMF-SOL was prepared (180 nM) in PBST at either pH 7.3 or 6.5 and analyzed immediately by TLC on Kieselgel $60F_{254}$ (0.2 mm thick) aluminum sheets (E. Merck) and an ethyl acetate/methanol/1% aqueous ammonia (80:20:1 v/v/v) solvent system (Plhak and Sporns, 1992) with UV visualization. The solutions were stored at room temperature in the dark for 30 days and reanalyzed by TLC.

Analysis of Potato Extracts by FPIA. GAs were extracted from potato samples as described by Plhak and Sporns (1992). Potato extracts were diluted sufficiently in methanol such that FP values of extracts were in the range of the standard curves for the major potato alkaloids (α-solanine, α-chaconine, and solanidine) following a final dilution in PBST. All potato extracts contained 10% (v/v) methanol when analyzed. Diluted potato extract (0.5 mL) was mixed with 0.5 mL of 180 nM AMF-SOL in PBST (pH 6.5) and 0.5 mL of a 1:66.7 dilution of PAb in PBST (pH 6.5). Individual GA contents were determined using the PAb FPIA standard curve for α-chaconine.

RESULTS AND DISCUSSION

Potato alkaloids and glycoalkaloids (GAs) are naturally occurring toxins in potatoes with potencies rivaling those of strychnine and arsenic (Morris and Lee, 1984). Breeding programs are often aimed at increasing disease and drought resistance of new cultivars; this often involves the use of wild potato varieties which generally contain exceedingly high levels of GAs (Van Gelder, 1991). It is therefore imperative to develop rapid and sensitive GA analysis methods for the screening of emerging potato cultivars.

Plhak and Sporns (1992) developed an indirect PAb-based competitive ELISA for the detection and quantitation of the major GAs in commercial potato cultivars. A MAb was also produced and found to be sensitive and specific for potato GAs in an indirect competitive ELISA (Plhak and Sporns, 1994). Despite the success of these two methodologies in detecting GAs, accurate quantitation continued to be a problem due to the high degree of variation associated with solid-phase IA results.

Our approach was to determine the feasibility of employing an FPIA method for the quantification of the major GAs in commercial potato cultivars using PAb and MAb using standard solutions of GAs. In addition, it was hoped that such a test could be applied to the analysis of potato extract material. Although the sensitivity of homogeneous IAs such as FPIA is inherently lower than that of heterogeneous IAs (Gosling, 1990), they are very reproducible and rapid and thus well-suited to the analysis of compounds present in large amounts (Eremin et al., 1991).

Characterization of AMF-SOL. The choice of fluorescein as the tracer molecule was based on its stability, in both its free and conjugated forms, high quantum yield, and ready excitability and detection of its fluorescence (Edwards, 1985; Gutierrez et al., 1989; Smith et al., 1981). 4′-AMF was selected to minimize the bridge length between the fluorescein molecule and the solanidine moiety (Figure 1) to favor strong competition between native GAs and the fluorescently labeled solanidine (AMF-SOL) (Colbert et al., 1991).

The combination of AMF-SOL with its specific PAb was found to reduce the fluorescence intensity of the fluorescein by approximately 14%, while the value was reduced 17% upon the interaction of AMF-SOL with the MAb, based on the ratio of the slopes of the regression lines of the standard curves for free and Ab-bound AMF-SOL. The decrease in fluorescence intensity is attributable to changes in the microenvironment surrounding the fluorophore. Jolley (1981) indicated that the fluorescence intensity of a tracer may increase, decrease, or remain unchanged upon binding to Ab.

While the addition of PAb solutions to AMF-SOL resulted in increased FP values, similar concentrations of PAb solutions and 4′-AMF did not give increased FP values. This indicated a lack of any nonspecific binding of the fluorescent group to Ab. The inclusion of Tween 20 in the buffer system probably also aids in preventing the nonspecific binding of the tracer and other immunoreactants to the test tube or cuvette walls.

Reducing the pH of PBST as the diluent for AMF-SOL from 7.3 to 6.5 did not affect FP values or wavelength maxima for excitation or emission; however, FI values decreased by 56%. Bieniarz et al. (1994) synthesized a phosphorothioate fluorescein molecule and indicated it had a pK_a value of 6.4; therefore, it is likely that the decrease of pH from 7.3 to 6.5 changed the ionization state of the fluorescein moiety of the AMF-SOL label. Such a change in ionization state would affect the resonance structure of the molecule and influence the fluorescent behavior of the molecule (Pesce et al., 1971).

The time required to reach equilibrium in the FPIA system was evaluated by mixing equal volumes of PAb and AMF-SOL to give a final dilution of 1:200 of PAb and a concentration of 60 nM in the FPIA and by reading FP values every 60 s (to 1 h) using a 10 s integration time. The time required to generate FP values precluded determination of the exact reaction time. As closely as could be determined, equilibrium was reached in 1−2 min of mixing immunoreactants. At room temperature, therefore, 30 min was more than a sufficient period of incubation.

Antibody Dilution Curves. A PAb dilution curve was prepared (Figure 2) to determine the antibody (Ab) concentration that netted a 20% reduction in the maximum FP value achieved with 57.5 nM AMF-SOL, using a protocol similar to those of Shipchandler et al. (1987) and García Sánchez et al. (1993).

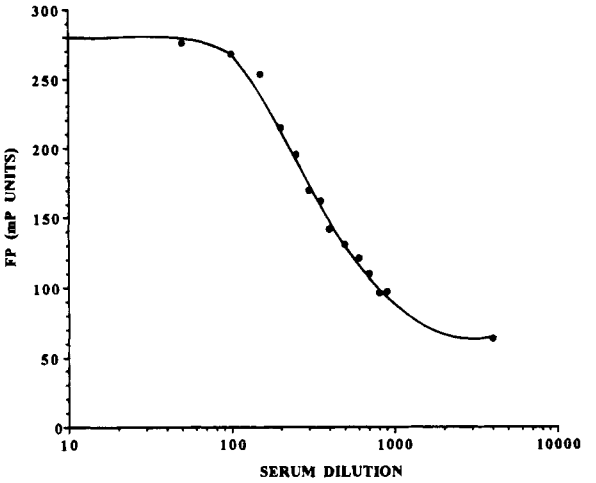

Figure 2. Polyclonal antibody dilution curve. Points represent the average of six determinations. Excitation wavelength was 496 nm (2.5 nm slit width); emission wavelength was 518 nm (7.5 nm slit width). Curve parameters, as determined using SOFTmax for original polarization data, were $a = 0.294$, $b = 1.71$, $c = 299$, $d = 0.062$, and $R^2 = 0.991$. Final label (AMF-SOL) concentration was 57.5 nM.

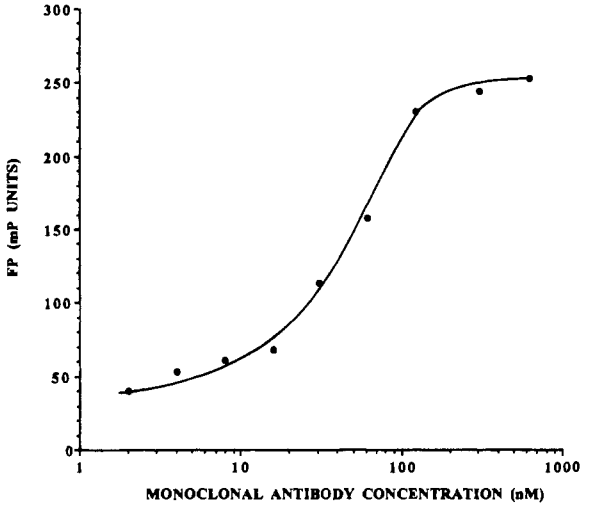

Figure 3. Monoclonal antibody dilution curve. Points represent the average of six determinations. Excitation wavelength was 496 nm (2.5 nm slit width); emission wavelength was 518 nm (7.5 nm slit width). Curve parameters, determined using SOFTmax for original polarization data, were $a = 0.052$, $b = 1.84$, $c = 53.7$, $d = 0.256$, and $R^2 = 0.983$. Final label (AMF-SOL) concentration was 60 nM.

A MAb dilution curve with a fixed amount of AMF-SOL is shown in Figure 3. For analysis, as noted for the PAb FPIA system, it was desirable to use a MAb concentration that gave approximately 80% binding of 60 nM AMF-SOL; this corresponded to a MAb concentration of 0.014 mg/mL (93 nM) in the FPIA reaction.

Kinetics of Ab-(AMF-SOL) Reaction with Polyclonal Serum and MAb. One of the drawbacks of working with a polyclonal serum is that it includes a population of different Abs, each with a different affinity for the analyte of interest. Moreover, it is exceedingly difficult to determine the concentration of Ab protein specific for hapten in such a preparation, as a variety of other non-Ig proteins and other Abs is present in a polyclonal serum (Coleman et al., 1989). Both of these factors constitute barriers to the determination of the kinetics of the reaction and necessitate an altered

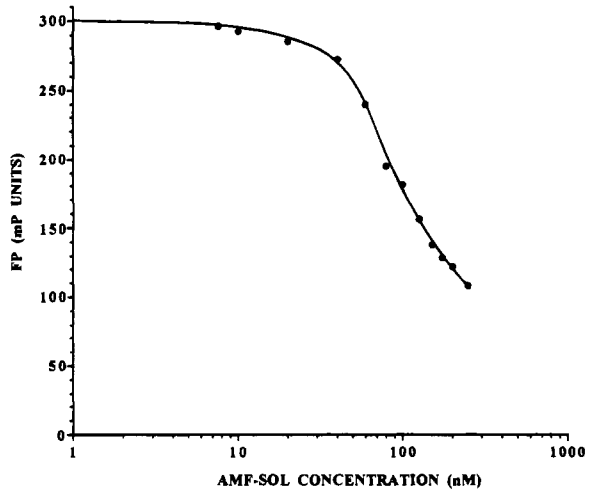

Figure 4. Label dilution curve with polyclonal antibody. Points represent the average of six determinations. Excitation wavelength was 496 nm (2.5 nm slit width); emission wavelength was 518 nm (7.5 nm slit width). Curve parameters, determined using SOFTmax for original polarization data, were $a = 0.296$, $b = 2.22$, $c = 88.4$, $d = 0.091$, and $R^2 = 0.993$. Final serum dilution was 1:200.

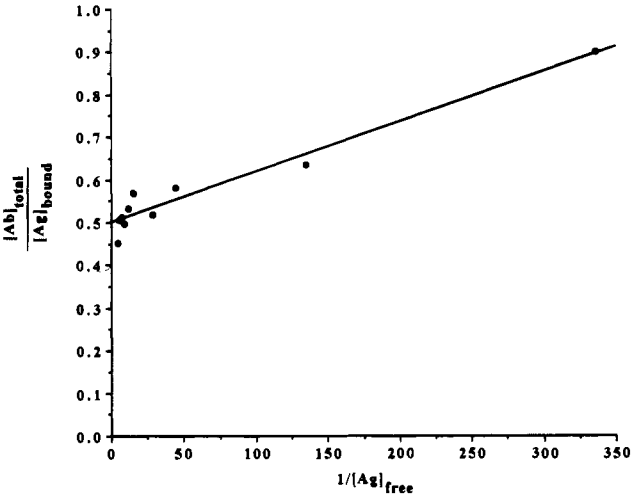

Figure 5. Langmuir plot for polyclonal antibody. Points represent the mean value of six determinations.

approach. In this study, an AMF-SOL dilution curve (7.5–350 nM) was prepared using a final PAb dilution of 1:200 (Figure 4). Using these data, and a number of assumptions (discussed below), it was possible to estimate an average affinity constant (K_{aff}) for the PAb population.

The Langmuir plot (van Oss and Absolom, 1984) allows for the determination of the kinetics of Ab reactions with an unknown but constant Ab concentration. The data obtained from the label dilution curve (Figure 4) were used to generate the Langmuir plot depicted in Figure 5. The percentage of label binding was calculated from

$$\frac{F_b}{F_f} = \frac{Q_f}{Q_b} \times \frac{(p - p_f)}{(p_b - p)}$$

where p = polarization reading, F = fraction bound (b) or free (f), Q_f = fluorescence of free tracer, Q_b = fluorescence of bound tracer, p_f = polarization value of free tracer, and p_b = polarization value of bound tracer (Dandliker and DeSaussure, 1970).

It was not possible to determine the lower asymptote of the label dilution curve due to the high FI values observed at very high AMF-SOL concentrations (Figure 4). Therefore, a "background" level of 46 mP units was estimated by measuring the FP of AMF-SOL in the absence of Ab.

A linear least-squares fit of the data was performed (Excel 4.0, Solver Macro) by varying the total concentration of Ab (the same for all y values) until the y intercept became 0.5. The Ab concentration that gave this intercept was 33 nM. To obtain a separate estimate of the amount of PAb present in diluted serum, Harlow and Lane (1988) was consulted. Their estimate of Ab found in the serum from a hyperimmunized rabbit was 10% of the 10 mg/mL present as IgG. Using this estimate, a MW of 150 000 (for IgG), and the final dilution of 1:200, the amount of PAb present was calculated to be 33 nM. Since both methods of estimating the concentration of PAb gave the same result, it was assumed that the data in Figure 5 could be used to calculate an average affinity constant (K_{aff}). The correlation coefficient for the straight line was 0.973, and the average K_{aff} calculated from the slope of the line (assuming all Ab was IgG; $n = 2$, MW = 150 000) was 4.2×10^8 M^{-1}. It should be stressed that this value for K_{aff} is a rough estimate of the general affinity of the polyclonal serum since it encompasses the affinities of a broad population of Abs for AMF-SOL.

The K_{aff} of the MAb was also determined using a Langmuir plot; however, in the case of the MAb, it was possible to vary the amount of MAb, as the concentration could be determined by UV spectrometry. The K_{aff} for Mab for AMF-SOL was estimated at 4.7×10^7 M^{-1}, which was approximately 1 order of magnitude lower than that for the PAb for the same label. This lower K_{aff} value for MAb may account in part for the approximately 3-fold increase in Ab requirements for the MAb system (93 nM) as compared to the PAb system (33 nM).

Detection of Potato Alkaloids and Alkaloid Derivatives Using PAb FPIA. The major potato GAs, solanidine, and compound **I** (solanidine hemisuccinate; Figure 1) were tested to assess the relative affinity of the PAb population for the individual GAs. The data in Figure 6 show that the PAb serum had the greatest affinity for α-chaconine, which was not surprising, considering that the polyclonal serum was raised against a derivative of chaconine. The PAb population had a similar but slightly reduced affinity for α-solanine, while the affinity of the serum for solanidine, the nonglycosylated potato alkaloid, was much reduced as compared to its glycosylated counterparts. These results for α-solanine, α-chaconine, and solanidine parallel the findings of Plhak and Sporns (1992), who examined the performance of the same PAb serum with GAs using a solid-phase enzyme immunoassay (EIA). α-Tomatine, a spirosolane GA, was not recognized by the polyclonal serum due to the difference in chemical structure from solanidine derivatives, particularly in the alkaloid portion of the molecule most responsible for Ab binding.

The PAb showed a lower affinity for the AMF-SOL tracer than for any of the major potato alkaloid components (Table 1) since 20 nM of any of the glycoalkaloids or alkaloids resulted in a greater decrease in FP values than did 20 nM label. That is, in the reaction containing the standard 60 nM concentration of AMF-SOL, the analytes tested were more successful in dis-

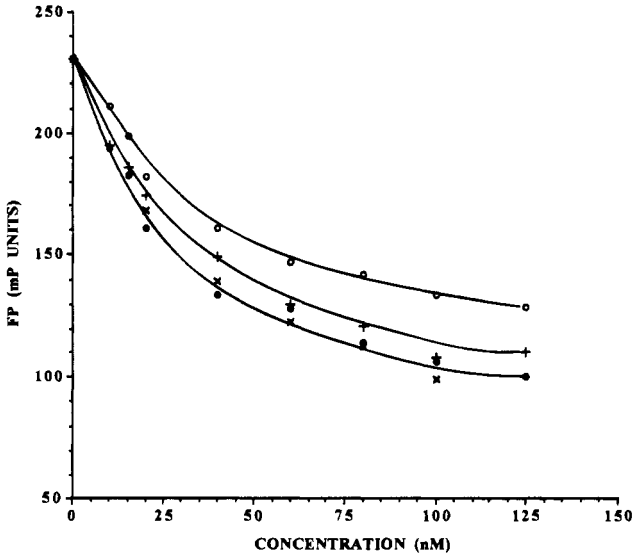

Figure 6. Standard curves for alkaloids and glycoalkaloids with polyclonal antibody. Points represent the average of six determinations; standard deviation of the mean was ≤9 mP units for all determinations. Excitation wavelength was 496 nm (2.5 nm slit width); emission wavelength was 518 nm (7.5 nm slit width). Label (AMF-SOL) concentration in reaction was 60 nM; serum was diluted 1:200. (○) Solanidine; (+) α-solanine; (●) α-chaconine; (×) compound **I**.

Table 1. Response of PAb to 20 nM GA or AMF-SOL

competitor	FP[a] (mP units)
α-chaconine	161
α-solanine	174
solanidine	182
compound **I**	168
AMF-SOL[b]	210

[a] Average (n = 6); standard deviations were ≤9 mP units for all determinations. All reaction mixtures contained 60 nM AMF-SOL label; final serum dilution was 1:200. Excitation wavelength was 496 nm (2.5 nm slit width); emission wavelength was 518 nm (7.5 nm slit width). [b] Label concentration was 80 nM (60 nM standard concentration + 20 nM for competition). Average maximum FP (no analyte) was 296.

placing the AMF-SOL from the Ab than was an equal concentration of the label itself.

Solanidine hemisuccinate (MW 498) and α-chaconine were equally well-recognized by PAb, which suggests that the addition of the fluorescein molecule itself reduces the affinity of PAb for AMF-SOL, as opposed to the presence of the hemisuccinate linkage. Since the Ab population was not produced in response to a compound containing fluorescein, there are no complementary binding groups on the Abs for the fluorescein moiety. The binding of the AMF-SOL by Ab may be influenced by steric effects and repulsive forces, which may negatively affect the binding of AMF-SOL to the Ab. The fact that the FI of the label was decreased upon binding to Ab further suggests interaction between the Ab binding site and the fluorescein moiety of the AMF-SOL label.

Ekins (1974) indicated that the sensitivity of an IA is optimized when the Ab displays equal affinity for both the analyte and the tracer molecule. Colbert et al. (1991) found that their PAb displayed greater affinity to the larger fluorescently labeled tracer molecule than for the analyte. They were able to reduce detection limits by decreasing the length of the linking arm to the fluorescent label, which permitted more comparable binding of both the analyte and the fluorescently labeled tracer by the Ab molecule.

Table 2. FP Values with Various Competitors and MAb in FPIA

[analyte] (nM)	fluorescence polarization value[a] (mP units)			
	α-chaconine	α-solanine	solanidine	compound **I**
20	205 ± 8	178 ± 3	215 ± 4	195 ± 5
40	139 ± 7	125 ± 8	176 ± 4	143 ± 8
60	107 ± 4	96 ± 1	153 ± 4	107 ± 6

[a] Average ± standard deviation (n = 6). Final monoclonal antibody concentration was 93 nM; label concentration in reaction was 60 nM. Excitation wavelength was 496 nm (2.5 nm slit width); emission wavelength was 518 nm (7.5 nm slit width). Average maximum FP (no analyte) was 253 mP units.

With this knowledge, we purposely used a fairly short succinyl group for linking the fluorescent label to the alkaloid. Obviously this interfered with the binding of tracer to Ab too much and favored the binding of analyte. Improved detection limits might be achieved by preparing a fluorescently labeled solanidine with a longer linking arm or perhaps a more rigid linking arm than the succinyl moiety.

Detection of Potato Alkaloids and Alkaloid Derivatives Using MAb FPIA. The evaluation of the FPIA system using MAb was similar to that for the polyclonal system, except that all evaluations were made at pH 6.5, as there was concern regarding the stability of the AMF-SOL label at pH 7.3. Subsequent TLC analysis indicated a limited amount (about 20%) of hydrolysis occurred at both pH 7.3 and 6.5 after an extended period of storage (30 days) at room temperature. This would not have affected the results discussed herein, however, as label dilutions in buffer were prepared fresh daily. The label concentration in all reactions was maintained at 60 nM.

The relative affinity of the MAb for α-solanine, α-chaconine, solanidine, AMF-SOL, and solanidine hemisuccinate was assessed at several levels of each competitor (Table 2). The MAb had the greatest affinity for α-solanine and comparable but slightly reduced affinity for solanidine hemisuccinate. The affinity for α-chaconine was slightly lower than that observed for α-solanine and solanidine hemisuccinate; however, it was much greater than that observed for solanidine. These findings, save for the hemisuccinate data which were not evaluated by Plhak and Sporns (1994), agree closely with those of the MAb EIA of Plhak and Sporns (1994).

Plhak and Sporns (1994), using the same polyclonal serum and MAb as were used in the present study, found that the PAb had a slightly higher affinity for GAs than did the MAb. The 17% decrease in FI values of AMF-SOL upon binding to Ab, as compared to the 14% observed for PAb, seems to indicate a greater degree of interaction between the AMF-SOL label and the MAb, likely due to differences in the configuration of the binding sites. The steric hindrance due to the fluorescent moiety may have significantly decreased the affinity of MAb for the AMF-SOL label.

Analysis of the Glycoalkaloid Content of Potato Samples. Three different potato extracts were analyzed for their GA content using the PAb FPIA system. Results, calculated using the regression line for the standard curve for α-chaconine, are presented in Table 3.

The potato samples analyzed were chosen to cover a wide range of GA concentrations; this is reflected in the values presented in Table 3. Results obtained for samples a-1, a-2, e-1, and e-2 were within 1 standard deviation of those obtained by solid-phase EIA by Plhak

Table 3. Glycoalkaloid Contents of Potato Samples Measured Using PAb

sample[a]	dilution of extract	polarization value[b] (mP units)	α-chaconine content[c] (mg/100 g, fresh weight basis) FPIA	EIA[d]
a-1	1:10	122 ± 4	1.6	1.2 ± 0.6
a-2	1:10	175 ± 4	0.5	1.2 ± 0.6
e-1	1:100	144 ± 7	9.7	9.9 ± 3.3
e-2	1:100	138 ± 3	11.1	9.9 ± 3.3
f-1	1:1000	149 ± 5	87.1	56.0 ± 10
f-2	1:1000	153 ± 7	79.8	56.0 ± 10

[a] 1 and 2 designate duplicate extractions. [b] Average ± standard deviation (n = 6). [c] All values calculated by assuming a 80% moisture content in fresh potatoes. [d] As reported by Plhak and Sporns (1992).

and Sporns (1992) for the same potato samples using the same PAb. Samples f-1 and f-2, however, were found to have much higher GA levels when analyzed by FPIA as compared to results achieved using an EIA, as reported by Plhak and Sporns (1992). This could be a reflection of the extraction technique employed, rather than a shortcoming of either IA method, as there is obviously some variation in methanol extraction efficiency (Table 3). The limitations of the simple methanolic extraction method for GAs are further illuminated by the fact that the standard deviation for FPIA results was very small and could not account for the large differences in α-chaconine levels observed in duplicate samples of potato material (Table 3).

An additional factor at work in causing variation in the values obtained from both the present study and that of Plhak and Sporns (1992) may have been the year interval between the original methanol extraction of freeze-dried material (Plhak and Sporns, 1992) and the second set of extractions (f-1, f-2). It is not uncommon to freeze-dry potato material to promote stability during storage such that samples may be analyzed at a later date (Bushway et al., 1985). Nonetheless, changes in the composition of the freeze-dried potato material during extended storage cannot be ruled out. It is unlikely that other materials in the potato matrix interfered significantly with the FPIA, since the most concentrated samples (a-1 and a-2, at a 1:10 dilution) gave results similar to earlier EIA and high-performance liquid chromatography analyses (Plhak and Sporns, 1992).

The cutoff value between safe and toxic potato tubers has been set at 20 mg/100 g of tuber (Van Gelder, 1991). In terms of the screening of new potato cultivars, it would be prudent to establish a cutoff value of 16 mg/100 g of tuber; that is, samples showing 16 mg/100 g of tuber should be reanalyzed. Using this value, and the FPIA system developed with PAb, sample extracts diluted 150 times and giving FP values ≤140 mP units should be reanalyzed.

Stability of PAb FPIA Standard Curve for α-Chaconine. One of the advantages cited for FPIA analysis is its intrinsic reproducibility, which extends to standard curves. These curves display long-term stability, simplify analysis, and reduce reagent requirements (Colbert et al., 1986; Thomas et al., 1991; Zaninotto et al., 1992). Furthermore, the use of stored calibration curves can improve the between-batch reproducibility of results (Thomas et al., 1991). In the present study, the stability of a standard curve for α-chaconine was monitored over 23 days. Reagents (Ab and AMF-SOL label) were stored separately over this time period and were mixed to allow for reaction on each day of analysis. Values did not change appreciably over the first 2 week period; however, mid-range values (20–60 nM) decreased when analyzed at 23 days.

The development of FPIA methods for GA analysis was contingent on the use of high levels of PAb or MAb, particularly when compared to the amounts required for a solid-phase assay (Plhak and Sporns, 1992). High serum concentrations (i.e., a dilution factor of <1000) have been employed by other investigators (Eremin et al., 1991; García Sánchez et al., 1993; Kobayashi et al., 1979; McGregor et al., 1978; Turner et al., 1991; Watson et al., 1976) and appear to be a necessary element in FPIA protocols.

Notwithstanding the increased serum demands of the FPIA systems described for GAs, the use of FPIA affords several advantages over solid-phase IAs. Since FPIA is a homogeneous technique, its attendant advantages include speed and the lack of a separation step, which permit automation of the analysis (Jenkins, 1992). If properly stored, the fluorescently labeled reactants also have a virtually indefinite shelf life (Smith et al., 1981), and the standard curves exhibit long-term stability, as has been discussed. Homogeneous assays, including FPIAs, are inherently less sensitive than heterogeneous assays (Eremin et al., 1991; Gosling, 1990). This was not specifically addressed in the current research, as extreme sensitivity is not essential in the analysis of potato samples since the levels of concern are quite high (Van Gelder, 1991).

ABBREVIATIONS USED

Ab, antibody; AMF-SOL, solanidine labeled with 4′-AMF; 4′-AMF, 4′-(aminomethyl)fluorescein; DCC, 1,3-dicyclohexylcarbodiimide; DMF, *N*,*N*-dimethylformamide; EIA, enzyme immunoassay; FPIA, fluorescence polarization immunoassay; FP, fluorescence polarization; GA, glycoalkaloid; IA, immunoassay; K_{aff}, antibody affinity constant; MAb, monoclonal antibody; mP, millipolarization units; PAb, polyclonal antibody; PBST, phosphate-buffered saline (0.1 M in phosphate; usually used at pH 7.3) containing 0.05% Tween 20; TLC, thin-layer chromatography.

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