Validation of an Enzyme Immunoassay for Analysis of Methoprene Residues on Tobacco

Robert A. Heckman,*,† Bruce S. Ferguson,‡ Titan S. Fan,‡ Joanne V. Mei,§,‖ Chih-Ming Yin,‖ Theodore R. Conner,† Vicki W. Addington,† and Herman J. Benezet†

Research and Development Department, R. J. Reynolds Tobacco Company, Box 2959, Winston-Salem, North Carolina 27102, ImmunoSystems, Inc., 4 Washington Avenue, Scarborough, Maine 04074, and Department of Entomology, University of Massachusetts, Amherst, Massachusetts 01003

Methoprene [isopropyl 11-methoxy-3,7,11-trimethyl-2(*E*),4(*E*)-dodecadienoate] is a synthetic insect growth regulator that is used extensively for control of the cigarette beetle (*Lasioderma serricorne*) in stored grains and tobacco products. In 1990 a successful cELISA was developed for methoprene; more recently, this immunoassay was redeveloped as an enzyme immunoassay (EIA). In the present study, HPLC data were used to test the accuracy and precision of the assay for use with various tobacco genotypes. Regression analysis of EIA results on HPLC values for the entire array of samples showed an intercept of 0.012 ± 0.506 and a slope of 0.926 ± 0.123. (*R,S*)-Methoprene was slightly less responsive than (*S*)-methoprene in the EIA. After several refinements were made, the assay was used by four analysts over several days. Replicate values for eight samples in the range 0–7.5 ppm gave an average coefficient of variation of 14%. The assay affords an attractive alternative for analysis of methoprene on tobacco, particularly in situations where chromatographic analysis is impractical.

INTRODUCTION

Methoprene [isopropyl 11-methoxy-3,7,11-trimethyl-2(*E*),4(*E*)-dodecadienoate] is a synthetic insect growth regulator that mimics the physiological functions of the insect juvenile hormones (Henrick et al., 1973). It is a potent and selective larvicide which interferes with the metamorphosis of insect larvae (Wakabayashi and Waters, 1985). It has also been found to be effective against the larval stage of a cyclopoidcopepod, *Cyclops vernalis* (Bircher and Ruber, 1988; Allen and Dickinson, 1990) which causes dracunculiasis.

Commercial formulations of methoprene are used for the control of a number of pests including mosquitoes, flies, ants, fleas, aphids, and stored-product pests. As the number of applications increases, so does the need for quantitative methods to detect methoprene residues. Many methods for detecting methoprene from various matrices have been developed. These methods include gas–liquid chromatography (GC) (Schaefer and Dupras, 1973; Miller et al., 1975; Kortvelyessy et al., 1984), high-performance liquid chromatography (HPLC) (Chamberlin, 1985; Heckman and Conner, 1989; Allen and Dickinson, 1990), and an infrared spectrophotometric procedure (Giang and Jaffe, 1980).

Methoprene is a "small" molecule (i.e., with a molecular weight <700) and will not elicit an immune response by itself. To raise antibodies against methoprene, it was covalently bound to a carrier protein for presentation to the mammalian immune system. The methoprene immunogen was designed such that a spacer group was incorporated between methoprene and the carrier. The spacer group was bound to methoprene through an ester and then to the carrier through an amide. Details regarding methoprene immunogen design and synthesis, antibody production, and development of an immunochemical assay for methoprene have been described previously (Mei et al., 1990, 1991). Polyclonal antisera have been raised in both mice (Mei, 1988) and rabbits (Mei et al., 1990) using a methoprene–spacer–protein conjugate as the immunogen. The polyclonal anti-methoprene antiserum from one rabbit was highly specific and did not cross-react with closely related compounds such as juvenile hormones (Mei et al., 1990), kinoprene, and hydroprene (Mei et al., 1991). Using these polyclonal antibodies, a competitive enzyme-linked immunosorbent assay (cELISA) and an enzyme immunoassay (EIA) have been developed which detect methoprene in water ranging from 5 to 300 ng/mL, with an I_{50} of 50 ng/mL (Mei et al., 1990), and from 1 to 10 ng/mL, with an I_{50} of 3–5 ng/mL (Mei et al., 1991).

This study describes (1) the use of an improved EIA which not only requires much less time to run than the cELISA but can also be used directly to detect methoprene residue from tobacco extract without the need of prior cleanup before analysis and (2) a correlation study comparing this EIA to an HPLC method which has been specifically designed for detecting methoprene residue in tobacco extract (Heckman and Conner, 1989).

MATERIALS AND METHODS

Chemicals. (*S*)- and (*R,S*)-methoprene were supplied by the Zoecon Corp. in stated purities of 90 and 94%, respectively. Other consumable supplies were provided by ImmunoSystems, Inc., Scarborough, ME, under the EnviroGard trade name and include the following items: tobacco extracts containing 0, 1, and 10 ppm of methoprene, methoprene–horseradish peroxidase enzyme conjugate, substrate, chromogen, stop solution, and strip holders containing eight anti-methoprene antiserum-coated strips of 12 wells each.

Instrumentation. HPLC analyses were conducted with a Varian Associates Model 5000 instrument configured to an AASP automated sample processor and Vista 402 data system as previously described (Heckman and Conner, 1989). Microplate readers were either Molecular Devices UV_{max} instruments configured to Macintosh or IBM personal computers or Bio-Tek Model EL311 configured to a Zenith PC; all three systems utilized 450-nm filters for strip/plate readings.

* Author to whom correspondence should be addressed.
† R.J. Reynolds Tobacco Co.
‡ ImmunoSystems, Inc.
§ Present address: Centers for Disease Control, 1600 Clifton Road F-19, Atlanta, GA 30333.
‖ University of Massachusetts.

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Enzyme Immunoassay (EIA) Procedure. Since detailed instructions accompany the assay materials (available from Millipore Corp., Bedford, MA) and microtiter strip preparation has been described in recent work with wheat (Hill et al., 1991), only a brief synopsis will be given here. Tobacco (1.0 g, ground) is extracted for 30 min with agitation using 24 mL of 90% (v/v) aqueous acetonitrile. A few milliliters of the resulting extract is filtered through a 0.45-μm filter. Standards, provided as 90% acetonitrile extracts, and samples are then diluted 1:20 by adding 100 μL of each to 2.0 mL of water. Diluted standards (80 μL) are then added to two microplate wells each. Diluted samples (80 μL) are added to duplicate wells also. Methoprene enzyme conjugate solution (80 μL) is added to each well in the same order. In all of these operations, time is of the essence. After the wells are covered with Parafilm and thorough agitation, the samples and standards are allowed to incubate at ambient temperature for 60 min, preferably with orbital shaking at 200 rpm. The contents of the wells are then shaken into a sink, followed by thorough rinsing of the wells under cool tap water. This emptying and rinsing cycle is repeated five times. Residual water is tapped out of the wells, followed by addition of two drops of substrate to each well. Chromogen (2 drops or 80 μL) is then added to each well following the same left to right order as above. The covered plates are allowed to incubate for 30 min, again with mixing on an orbital shaker. "Stop" solution (1 drop or 40 μL of 2.5 N H_2SO_4) is added to each well following the same order of addition. Wells that appeared in varying shades of blue prior to this step will then become varying shades of yellow. After thorough mixing, the strips/plates are read on a microplate reader equipped with a 450-nm filter. Results are obtained using system software. Alternatively, results for unknowns may be determined using three-cycle semilog paper by plotting log standard concentration vs absorbance.

Later Refinements. Following extensive testing by additional analysts, it was determined that the assay was improved by making two changes in the above protocol. These were (1) 80% (v/v) aqueous methanol was substituted for aqueous acetonitrile and (2) 100-μL quantities of diluted samples, standards, and methoprene enzyme conjugate were used vs 80 μL of these same materials. Also, whereas the prototype assay utilized (*S*)-methoprene (the biologically active isomer) as standard, later materials and those now available commercially contain (*R,S*)-methoprene.

Validation Protocol. Samples of ground tobacco that had previously been analyzed by HPLC (Heckman and Conner, 1989) were submitted without knowledge of previous results for EIA at the development laboratory (Immunosystems, Inc.). EIA results were received by fax and compared by linear regression plots. All of these comparative studies involved mean values for duplicate HPLC results; triplicate well analyses from single tobacco extracts were used for EIA.

(*S*)- vs (*R,S*)-Methoprene. In all of the early validation work, (*S*)-methoprene was used as standard. To compare the responsiveness of a racemic standard in the EIA vs the *S* enantiomer, flue-cured tobacco extracts were fortified with 5 ppm of each material. For this work, freshly prepared standards containing (*S*)-methoprene were used that represented 0.5, 1.0, 5.0, and 10.0 ppm. Several analyses of *S*- and *RS*-fortified extracts (5 ppm each) were performed in quadruplicate using four-point semilog calibrations.

RESULTS AND DISCUSSION

EIA data together with HPLC values were used to prepare scattergrams and regression statistics. In these early comparisons, EIA data were arbitrarily chosen as the independent variable. Emphasis was placed on evaluating EIA performance on each of three major genotypes individually. Standards for analysis of flue-cured/oriental tobaccos utilized flue-cured tobacco extract, whereas burley tobacco extract was used for burley tobacco types. Results for these small sets of challenge samples are shown in Table I.

To rigorously compare the two analytical methods, all of the data points in Table I were pooled using values obtained by the established HPLC method as the independent variable (precision for the HPLC method was 2.6% CV at the time of validation). The unweighted regression line technique (Miller and Miller, 1984) was applied to the consolidated data set. Perfect agreement between the two methods would yield a regression line having a zero intercept (a) and a slope (b) and correlation coefficient (r) of 1. A test was made for an intercept differing significantly from zero and a slope differing significantly from 1 by determining the confidence limits for a and b at the 95% significance level. The appropriate t value (2.04) for 32 ($n - 2$) degrees of freedom, together with the sample standard deviations of a and b, afforded the 95% confidence limits for the intercept and slope shown:

$$a = 0.012 \pm 0.506$$

$$b = 0.926 \pm 0.123$$

The calculated slope and intercept did not differ significantly from the ideal values mentioned above. Hence, there was no evidence at this point for systematic differences between the HPLC and EIA methods. There was also no evidence for serious cross-reactivity problems involving tobacco extractives in the EIA method. Obviously, random errors can occur in both analytical methods. However, this regression approach assumes that all errors occur in the y (EIA) direction. The r value of 0.938 for the 34-sample set suffered somewhat by comparison with r values for the subsets. This increase in variation for the pooled data was not surprising and suggested a lack of precision for either one or both of the methods. Also, a graph of the regression line showed that most of the variation from the line occurred at higher concentration levels. Such an increase in absolute error with increasing concentration is typical of most methods. The limit of detection (LOD) in this assay is readily estimated by adding a value of 3 times the average standard deviation for the blanks in Table II to the EIA value (0.4) obtained for both blanks. This yielded an LOD of 0.64 ppm; the upper limit is 10 ppm.

In fact, extended use of the kits did reveal disappointing precision with CVs sometimes in excess of 20%. Also, EIA values were somewhat depressed relative to known residue levels on control samples. The precision aspect was dealt with successfully by incorporating the refinements outlined under Materials and Methods. The negative bias was corrected by substituting (*R,S*)-methoprene for (*S*)-methoprene as standard.

Table I. Linear Regression of HPLC Values on EIA Results for Major Tobacco Types

genotype	n	range, ppm	slope	intercept	r
flue-cured	8	0.0–7.8	1.17	–0.191	0.985
flue-cured	6	0.7–8.3	1.37	0.436	0.975
burley	6	0.6–8.0	0.77	–0.051	0.995
burley	9	0.0–7.4	1.07	0.224	0.990
oriental	5	0.0–7.4	1.03	0.150	0.995

Table II. Precision of the EIA during Extended Use

sample	HPLC value, ppm	mean EIA value, ppm	n	s	% CV
1	<0.15	0.4	6	0.047	12
2	6.8	9.5	6	0.65	7
3	5.2	7.7	6	1.2	16
4	7.5	9.4	6	0.79	8
5	3.9	4.2	6	0.28	7
6	<0.15	0.4	8	0.11	29
7	2.1	2.4	8	0.47	20
8	5.8	6.4	8	1.1	17

Assay of the (*R*,*S*)-methoprene extract on three occasions yielded values of 4.8, 4.2, and 4.8 ppm. Comparable results for the extract fortified with (*S*)-methoprene were 6.6 and 5.7 ppm (one sample set was ruined). Thus, there appears to be some difference in responsiveness between optically active methoprene and the racemic modification in the EIA that was not revealed during validation. However, the difference seems to be much less than a factor of 2. Possibly this can be attributed to the presence of (*R*)-methoprene as an impurity during the antibody development stage of the assay. This stereochemical distinction has practical ramifications as well, since Kabat (the brand of methoprene sold as insect control agent for tobacco and stored grains) contains the racemic modification, whereas Dianex (sold to control infestation of work spaces) has been recently formulated with the *S* enantiomer.

After the assay was reformated, it was evaluated through extended use in two laboratories by four analysts over several workdays. The results of this effort are summarized in Table II. The test samples were a mix of flue-cured and burley types, and the same standards were used for all samples, regardless of genotype. Of particular interest were the coefficients of variation (CV) that resulted from this ruggedness test. The average value of 14% should be adequate for a screening method and particularly for use in situations where chromatographic analysis is not feasible.

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