Phytochemistry, 1981, Vol. 20, pp. 343–344. © Pergamon Press Ltd. Printed in England. 0031-9422/81/0201-0343 $02.00/0

A SENSITIVE METHOD FOR DETECTION AND QUANTITATIVE DETERMINATION OF PYRROLIZIDINE ALKALOIDS

H. BIRECKA, J. L. CATALFAMO and R. N. EISEN

Department of Biological Sciences, Union College, Schenectady, NY 12308, U.S.A.

(*Received* 10 *March* 1980)

Key Word Index – Pyrrolizidine alkaloid; detection; quantitative determination; methyl orange.

Abstract—A sensitive and rapid method for detection and quantitative determination of pyrrolizidine alkaloids is presented. It is based on stoichiometric reaction of protonated alkaloids with methyl orange, followed by a release of the latter from the complex. The color intensity of the dye is assessed visually or spectrophotometrically. The method easily detects alkaloids at a concentration of 0.5 μg/ml. The sensitivity of the quantitative assay ranges from 0.006 to 0.045 μmol/ml (from about 2 to 15 μg/ml). The method proved to be especially useful during extraction, purification, and separation of minor pyrrolizidine alkaloids.

INTRODUCTION

The Dragendorff reagent in various modifications [1, 2] and titration with 10 mM *p*-toluenesulfonic acid in chloroform [3] have been commonly used for detecting and estimating pyrrolizidine alkaloids (PA's), respectively. The Dragendorff reagent shows a relatively low sensitivity to saturated as well as unsaturated PA's; spots on paper of about 10 mm^2 containing less than 3 μg of PA's, are hardly detectable. A reliable titration even with 1 mM sulfonic acid requires well over 30 μg of PA's. The highly sensitive Mattocks' method [4, 5] allows one to assay only unsaturated PA's and is time-consuming.

A wide range of dyes has been used for spectrophotometric determination of alkaloids. Methyl orange was first introduced for estimation of cinchona alkaloids [6]. Modifications of the methyl orange reaction have been used for quantitative determination of various alkaloids, in particular nicotine, in human organs [7] or quinolizidine alkaloids in plants [8, 9]. In this study further modifications of the methyl orange method for detection and determination of PA's are described.

RESULTS AND DISCUSSION

The methods described are based on protonation of PA's in $CHCl_3$ solution with boric or acetic acid and their stoichiometric reaction with aqueous methyl orange; a yellow complex highly soluble in $CHCl_3$ is formed. Methyl orange is released from the complex in $CHCl_3$ by H_2SO_4 in ethanol after separation of the aqueous medium; its color intensity, assessed visually or measured at 525 nm, is indicative of the PA concentration.

Detection and visual assessment of PA's

Various PA's, containing heliotridine, retronecine, supinidine, or trachelanthamidine as the necine moiety, were tested either separately or in mixtures. When reagent I (methyl orange and boric acid in H_2O) is used at 10 μl/ml $CHCl_3$, a PA concentration of about 0.5 μg/ml $CHCl_3$ can be readily detected. The presence of residual pigments in petroleum, ethyl ether, or chloroform extracts of PA's from plant material did not alter the sensitivity. Neither did traces of NH_3 in the solvent interfere with the detection. The upper limit of a semiquantitative visual assessment is about 15 20 μg PA/ml. Solvents, in particular methanol, in which aqueous methyl orange may be soluble, must be removed before $CHCl_3$ is added. When used for determining the completeness of extraction or the pattern of PA separation during liquid chromatography on various types of columns, the method proved to be much more reliable than testing with Dragendorff reagent. Its high sensitivity is of special importance in cases of minor PA's occurring in trace amounts in plants.

Quantitative determination

When reagent I, pH 5.2, was used for spectrophotometric determination, significant differences in the molar absorptivity were found between various alkaloids (Table 1). These differences seem to be due to variations in the dissociation constants of the latter. Protonation of PA's with 0.5% acetic acid, pH 2.9, at 5 μl/ml $CHCl_3$, followed by addition of methyl orange solution at 5 μl/ml $CHCl_3$, decreased the differences. However, similar values for various PA's including their *N*-oxides were obtained only when 1.25% acetic acid, pH 2.7, at 2 μl/ml $CHCl_3$, followed by addition of methyl orange at 5 μl/ml, was applied. The molar absorptivity indicated for acetylcurassavine, detected in *Heliotropium* plants [11], is similar to that found with other trachelanthamidine-containing PA's (to be published). Free necines, e.g. heliotridine, could not be determined even using 1.25% acetic acid and reducing the total amount of H_2O to 7 μl/ml.

The molar absorptivity values were obtained within the range of PA concentrations from 0.006 to 0.045 μmol/ml, e.g. from 2 to 15 μg of monocrotaline per ml $CHCl_3$. At higher concentrations the values are lower, apparently due to an insufficient concentration of methyl orange (saturated 0.5% solution) as a reactant. Lower molar absorptivities were also obtained when the dye was premixed with the acetic acid solution. Intensely colored

Table 1. Calibration data for some pyrrolizidine alkaloids. Absorbance (A) measured at 525 nm

Alkaloid	MW	pK_a*	A ($mg^{-1} ml^{-1}$)			Molar absorptivity		
				CH_3COOH			CH_3COOH	
			H_3BO_3	0.5%	1.25%	H_3BO_3	0.5%	1.25%
Lasiocarpine	411	6.55 (7.64)	0.059	0.087	0.106	24 249	35 757	43 566
Monocrotaline	325	6.93	0.082	0.118	0.131	26 650	38 675	42 575
Heliotrine	312	7.82 (8.52)	0.102	0.126	0.132	31 824	39 312	41 184
Supinine	283	8.44 (9.58)	0.146	0.150	0.152	41 318	42 450	43 016
Lasiocarpine *N*-oxide	427		0.071	0.082	0.096	30 181	35 018	40 992
Supinine *N*-oxide	299		0.095	0.125	0.142	28 405	37 375	42 458
Acetylcurrassavine	341		0.124	0.124	0.127	42 284	42 284	43 307

*pK_a values determined by Culvenor and Willette [10] in 80% methyl-cellosolve/water or in H_2O (in parentheses).

samples may be diluted with acidic $CHCl_3$ (10 parts of $CHCl_3$ and 1 part of reagent III–H_2SO_4 in ethanol). The rapid semiquantitative assay here described can be helpful in adjusting the concentration of unknowns to the sensitivity range.

The modified methyl orange method, with a sensitivity comparable to that of Mattocks' method, is much less error-prone, more rapid, and less expensive than the latter. It does not, however, allow one to distinguish unsaturated PA's from saturated ones. The method is much more sensitive than the titrimetric method; it was also much less affected by impurities when tested using PA extracts at various stages of purification.

For detection and visual assessment of PA's, reagent I proved to be quite adequate. Unlike the quantitative method, the semiquantitative one does not require centrifugation for phase separation.

EXPERIMENTAL

Reagents. Reagent I: solution A – 500 mg finely powdered methyl orange is dissolved in 100 ml H_2O at 40° for 20 min, cooled to 20° and filtered; solution B – 12 g of H_3BO_3 in 100 ml H_2O at 100°, cooled to 20°, and the saturated solution decanted. Reagent I is prepared by mixing together both solutions in a 1:1 ratio; in order to prevent precipitation, prepare fresh. Reagent II: 1.25% aq. HOAc. Reagent III: 2 ml conc. H_2SO_4 is dissolved in 100 ml EtOH. All chemicals used, including $CHCl_3$, are reagent grade.

Procedures. Detection and visual assessment of PA's: 5–100 μl (or more) of a PA solution are transferred to a 10 × 100 mm test tube. If necessary, the solvent is evapd directly from the tube by vacuum. 1.0 ml of EtOH-free (dried) $CHCl_3$ is added, followed by 10 μl of reagent I. The solns are vigorously hand-mixed for 10 sec. After 1–2 min standing, the phases separate; 0.5 ml of the $CHCl_3$ phase is carefully removed and transferred to a dry tube. Addition of 50 μl of reagent III followed by mixing, releases methyl orange from the PA complexes. Its concentration is assessed visually. Semiquantitative estimation is facilitated by comparing the color intensity with standard solutions of a PA at three concentrations between 1 and 15 μg/ml in capped tubes. The blank should be colorless.

Quantitative determination. 25–500 μl (or more) of a PA solution are taken and if necessary, the solvent is evaporated by vacuum. 5 ml dried, EtOH-free $CHCl_3$ followed by 10 μl of reagent II are added and the solutions, in sealed vials, are mechanically mixed at high speed for 5 sec. Immediately afterwards, 25 μl of solution A (see reagent I) are added and the mixing procedure is repeated for 10 sec. The phases are allowed to separate by standing 1–2 min; 3–4 ml of the $CHCl_3$ phase is taken and centrifuged at about 400 × g for 2 min. 1.5–3.0 ml of the $CHCl_3$ phase is then transferred and reagent III is added (0.1 ml/ml), the solns mixed, and the absorbance is measured at 525 nm versus a colorless blank.

Acknowledgement – We are grateful to Dr. C. C. J. Culvenor for his gift of pure pyrrolizidine alkaloid samples. Monocrataline was a gift from S. B. Pennick & Co. Lyndhurst, N.Y.

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