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Note

Fluorimetric and high-performance liquid chromatographic determination of harmane alkaloids in *Peganum harmala* cell cultures

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Cultured cells, even when grown in one flask, are known to differ greatly in their biochemical potential for producing secondary compounds[1]. It has been shown that highly productive cell clones can be selected from wild-type populations by analytical means[1,2]. An analytical selection method should be as simple and as sensitive as possible for screening a large number of small samples in a short time. In connection with studies on cell cultures of *Peganum harmala*, it became necessary to establish a method for screening cell colonies for their harmane alkaloids contents.

According to Gröger[3], *Peganum* plants contain the harmane derivatives harmine, harmol, harmaline and harmalol, which can be divided in two groups according to their oxidation stage (Fig. 1).

The quantitative determination of the alkaloids in extracts by measuring their UV absorption is possible only after separation[4,5] and is not very sensitive. However, harmane alkaloids are fluorescent compounds with characteristic emission spectra. This fact was used to establish a rapid method for distinguishing between both groups of alkaloids in the same unpurified cell extract. To quantitate all four harmane derivatives of cell culture extracts the alkaloids were separated by high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Materials

Alkaloids were obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals were purchased from different commercial sources. Cell cultures of *Peganum harmala* were initiated from different organs of sterile grown seedlings. The cultures were maintained on a Murashige–Skoog medium[6] with 1.8 μM 2,4-D. Methanolic cell extracts (25 mg dry weight per 25 ml) were centrifuged, filtered through a 0.45-μm cellulose filter and diluted with methanol or water.

Fluorescence measurement

A Jobin Yvon JY 3D spectrofluorimeter with excitation and emission monochromators was used.

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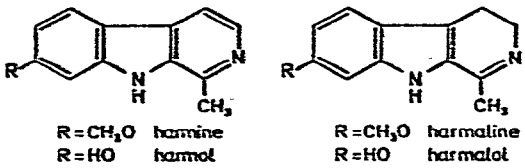

Fig. 1. The alkaloids of the harmane group.

High-performance liquid chromatography

An LDC Model II G Constametric pump in conjunction with a Rheodyne Model 7120 syringe-loading injector was used. Detection was effected by means of an LDC Model 1204 spectromonitor III set at 330 nm and a JY 3D spectrofluorimeter. Chromatography was carried out using a 25-cm Merck RP-8 column (7 μm) combined with an RP-2 pre-column (30 μm) and isocratic elution with methanol–water–formic acid (166:34:1) buffered with triethylamine at pH 8.5. The flow-rate was 1 ml/min, and the column pressure was 1670 p.s.i.

RESULTS AND DISCUSSION

Fluorimetric determination

The fluorescence spectra of the four harmane alkaloids are shown in Fig. 2. In methanolic solution harmine and harmol had nearly the same maximum at 355 nm with optimal excitation at 304 nm. Harmaline and harmalol showed no emission under these conditions. They fluoresced maximally at 475 nm, when excited at 396 nm, whereas in this instance harmine and harmol did not show any fluorescence. The spectra of harmaline and harmalol were similar. The different fluorescence spectra of harmine/harmol and harmaline/harmalol allowed the determination of the two groups of alkaloids in the parts per billion (10^9) range without separation.

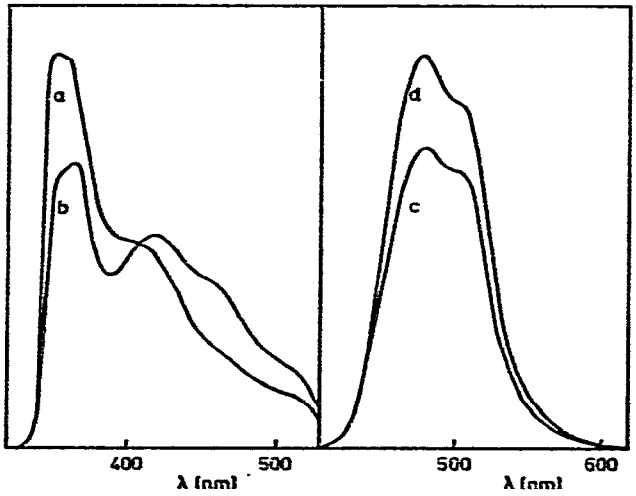

Fig. 2. Fluorescence spectra of harmine (a), harmol (b), harmaline (c) and harmalol (d) in methanol. Excitation at 304 nm (a,b) and 396 nm (c,d).

In the crude *Peganum* cell extracts tested, the fluorescence at 355 nm was masked by that of other compounds. However, dilution with water led to bathochromic shift of the fluorescence spectra of harmine and harmol (Fig. 3). Thus, solutions with a water to methanol ratio of 9:1 and higher did not fluoresce at 355 nm. The maximum shifted to 425 nm with optimal excitation at 324 nm. In this range other compounds of the cell extract did not interfere. The fluorescence spectra of harmaline and harmalol were not altered by dilution with water. Quenching effects were determined by measuring the fluorescence increase after the addition of a known amount of the alkaloid to a sample and comparing it with the expected value.

Changing the pH of the solution did not alter the fluorescence spectra, but the emission intensity decreased in solutions above pH 6.5. Therefore, the extracts were diluted with phosphate buffer (0.2 *M*, pH 5.0) to exclude errors caused by pH variations. The described conditions provided a simple method for screening a large number of samples for their harmane alkaloid contents. The relative standard deviation of rapid assays, *i.e.*, extraction with methanol, dilution with buffer and measuring the fluorescence with internal standardization, was less than 10%. Standard solutions of harmine and harmaline were prepared and the measured fluorescence values were expressed as harmine and harmaline equivalents. Systematic errors were thus introduced, because the emission intensity of harmine is higher than that of harmol, but it was negligible to purpose of our investigations, as in *Peganum* cultures the harmol concentration was only 5% that of harmine. Harmaline and harmalol were equal in intensity. Calibration graphs for harmine (425 nm) and harmaline (475 nm) were linear up to 1 and 3 μg/ml, respectively. The detection limit was below 0.5 μg/ml, at which concentration the signal-to-noise ratio was 4.

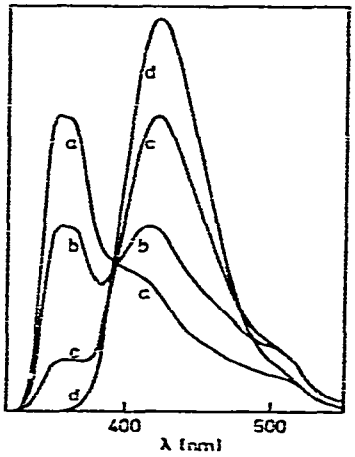

Fig. 3. Shift of harmine fluorescence with variation in the proportions of methanol and water in the solution (a, 10:0; b, 9:1; c, 7:3; d, 1:9). Excitation at 304 nm.

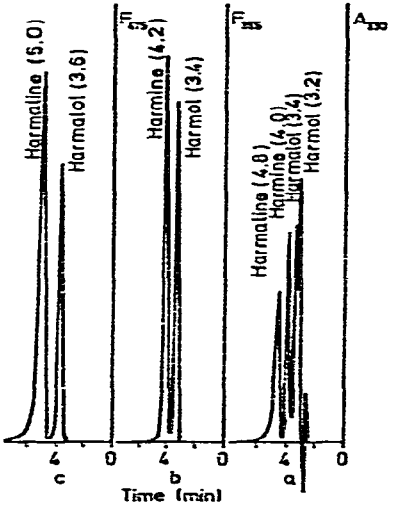

Fig. 4. HPLC separation of a standard mixture of harmane alkaloids on a reversed-phase column. Conditions as described in the text. Detection was effected by measuring absorbance (a, 330 nm) and fluorescence (b, 355 nm, excitation 304 nm; c, 475 nm, excitation 396 nm). Retention times (minutes) are given in parentheses.

High-performance liquid chromatography

Fig. 4 shows the separation of harmol, harmalol, harmine and harmaline. All four alkaloids could be detected by measuring the absorbance at 330 nm. This wavelength was chosen for measuring the four alkaloids in one chromatographic run. In methanol solution the absorbance maximum of harmine and harmol is at 240 nm and that of harmaline and harmalol at 380 nm. As for the fluorescence spectra, we found a bathochromic shift of the absorbance spectra of harmine and harmol in aqueous solutions. Using the above fluorimetric systems a better quantitative measurement was possible. Furthermore, fluorimetric determination increased the sensitivity by 100-fold. The detection limit was below 10 pg. For both methods the calibration graphs showed linear regression coefficients between 0.993 and 0.99998 in a tested range from 1 to 200 ng.

Fig. 5 shows a chromatogram of a methanolic extract of a *Peganum harmala* suspension culture. Harmalol, harmine and harmaline were clearly detected. However, at 355 nm we found another peak due to an unknown compound with a retention time 0.4 min less than that of harmol, which masked the small harmol signal. On measuring the above fluorescence maximum of harmine and harmol at 425 nm, the

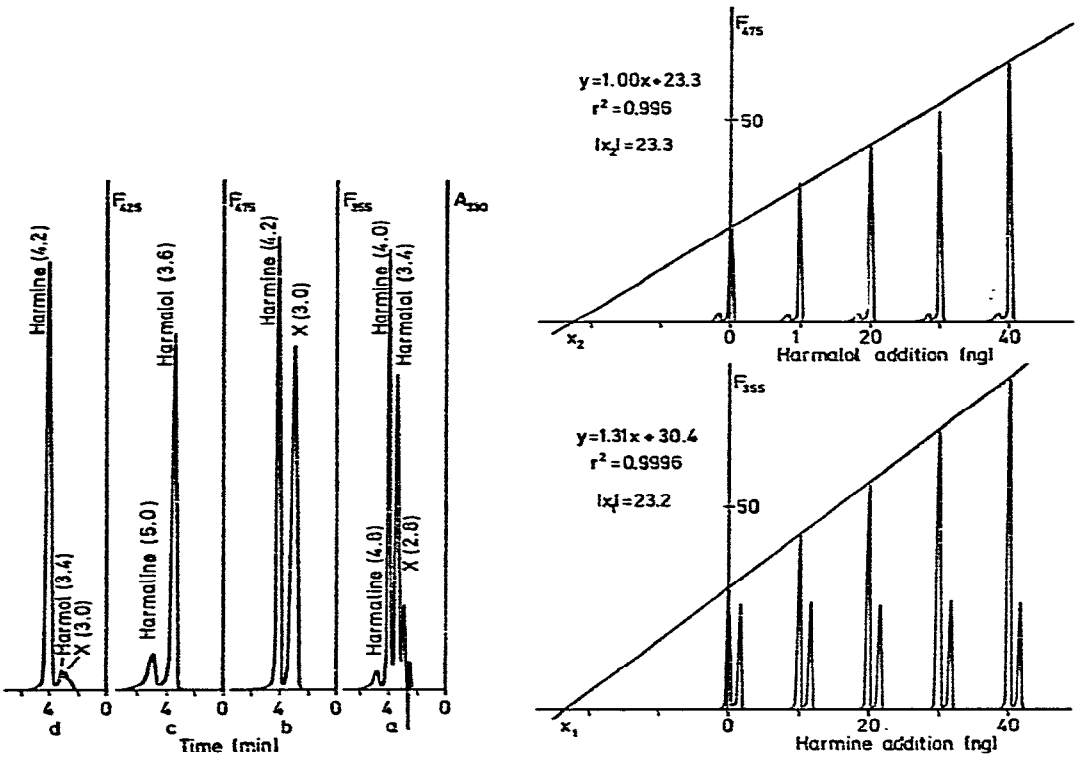

Fig. 5. Fluorimetric detection of HPLC-separated methanolic extract of a cell culture of *Peganum harmala*. Chromatographic conditions as in Fig. 4. Detection: a, absorbance (330 nm); b, c and d, fluorescence (b, 304/355 nm; c, 396/475 nm; d, 324/425 nm). Retention times (minutes) are given in parentheses.

Fig. 6. Determination of harmine (a) and harmalol (b) by internal calibration.

size of the unknown peak decreased considerably, and the small harmol signal could be detected.

The HPLC-separated alkaloids were quantitatively determined by internal calibration. Thus, the same conditions for the standard and the compound to be measured were provided. Fig. 6 shows the determination of fluorimetrically detected harmalol and harmine. It also provides proof of the identity of the measured compounds. The two other alkaloids could also be identified in this way. For routine determinations external calibration is preferable.

Peganum cells cultured on a standard medium contained mainly harmine and harmalol, and only trace amounts of harmol and harmaline. This is in agreement with results found by Nettleship and Slaytor[5], while in other *Peganum* cultures only harmine was detected[4].

The alkaloid pattern in seeds is different, harmaline and harmine being the predominant alkaloids[3,7]. The alkaloid patterns of intact plants seem to vary in a wide range[3–5], but harmine is always the main compound and harmalol is of lesser importance.

A comparison of the results obtained by determining the alkaloids in crude extracts and after HPLC showed good correspondence between the two methods and provided proof of the practicability of the simple fluorimetric method when screening of cell lines is needed. The determination of the individual alkaloids by HPLC is possible.

ACKNOWLEDGEMENTS

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