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Analysis of tropane alkaloids with thermospray high-performance liquid chromatography–mass spectrometry

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ABSTRACT

A thermospray high-performance liquid chromatography–mass spectrometry method for analysis of hyoscyamine and scopolamine in plant cell culture samples is described. The alkaloids were separated on a polymeric reversed-phase column with an alkaline ammonium acetate buffer–acetonitrile eluent. Selected-ion recording of the protonated molecular ions was used for quantitation of the compounds. The compounds were fragmented by discharge-assisted ionization and elevated thermospray capillary temperatures or ion repeller potentials.

INTRODUCTION

Various *Solanaceus* plants, such as *Atropa belladonna* and *Hyoscyamus niger*, produce tropane alkaloids [1]. Two of these alkaloids, atropine [(±)-hyoscyamine] and scopolamine (see Fig. 1), have been used in clinical applications for a long time [2].

Several chromatographic methods have been developed for the analysis of tropane alkaloids. The pharmacokinetic studies have been based on gas chromatography–mass spectrometry (GC–MS) after trimethylsilylation [3] or after hydrolysis and formation of heptafluorobutyryl derivatives [4]. GC has also been applied in analysis of tropane alkaloids in cell culture samples [5,6].

Several high-performance liquid chromatographic (HPLC) methods have been developed for the analysis of tropane alkaloids in plant and pharmaceutical samples. Scopolamine and hyoscyamine are weak bases with pK_a values of 8.2 and 9.7, respectively [7]. Thus the ion-pair HPLC technique may be used to achieve better peak-shape and separation of the compounds. The method has

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been applied to analysis of atropine in pharmaceuticals using alkylsulphonates or cyclamate as the ion–pairing reagent [8]. An ion-pair HPLC technique using 17.5 m*M* sodium dodecyl sulphate as the counter-ion in a mobile phase of phosphate buffer–acetonitrile has been successfully applied for the analysis of several tropane alkaloids in plant material after simple sample treatment [9,10].

Thermospray HPLC–MS has proved effective for the detection of various polar alkaloids, for example strychnine [11], indole alkaloids [12] and ergot alkaloids [13]. It has also been effectively used in identification of eight tropane alkaloids in plant cell cultures [14].

Use of a thermospray source with buffer ionization usually provides a means of obtaining molecular adduct ions with little or no fragmentation. The fragmentation may be achieved in several ways, including temperature variation of the vapourizer and variation of the ion repeller electrode potential [15,16].

This paper describes the application of a poly(styrene–divinylbenzene) copolymer as a stationary phase in the HPLC analysis of hyoscyamine and scopolamine in plant cell culture samples. This packing material allows the use of alkaline eluents, where weak bases appear neutral [17]. Thus the use of ion-pairing reagents is not required, which makes the mobile phase suitable for thermospray HPLC–MS analysis. Quantitation was completed by selected-ion recording (SIR) of the protonated molecular ions. Fragmentation was accomplished by discharge ionization and a higher thermospray capillary temperature or an increased ion repeller potential.

EXPERIMENTAL

Chemicals and sample preparation

Scopolamine hydrobromide and homatropine hydrobromide were purchased from Aldrich (Milwaukee, WI, U.S.A.) and hyoscyamine hydrobromide from Sigma (St. Louis, MO, U.S.A.).

The samples consisted of 10–50 mg of cultured plant cells originating from the genera *Duboisia* and *Hyoscyamus*. The samples were extracted and prepurified as described by Ylinen *et al.* [6], but homatropine was used as an internal standard instead of ketamine and the alkaloids were dissolved in 1.0 ml of methanol instead of methylene chloride before HPLC injection.

HPLC conditions

The HPLC system consisted of a Beckman Model 110B pump (Beckman Instruments, San Ramon, CA, U.S.A.) and a Rheodyne 7125 injector (Rheodyne, Cotati, CA, U.S.A.) with a 20-μl loop. The column was a PRP-1 (150 × 4.1 mm I.D., 5 μm particle size; Hamilton, Reno, NE, U.S.A.) polymeric reversed-phase column. The isocratic eluent consisted of 0.1 *M* ammonium acetate (pH 10.4)–acetonitrile (70:30). The flow-rate was set to 1.0 ml/min.

Mass spectrometry

The HPLC–MS system used was a VG thermospray–plasmaspray probe coupled to a VG Trio-2 quadrupole mass spectrometer (VG Masslab, Manchester, U.K.). The quantitative measurements were taken with the instrument in the thermospray mode. The ion source temperature was 150°C, the vaporizer tip temperature 170°C and the repeller electrode potential 150 V. The thermospray spectra were recorded for 2 μg of alkaloid chromatographed as described above. SIR was based upon the protonated molecular ions: *m/z* 304 for scopolamine, *m/z* 290 for hyoscyamine, and *m/z* 276 for the internal standard, homatropine.

Discharge ionization studies were performed using a 600-μA current. The spectra were obtained by injecting 20 μg of the compounds via a loop. The ion source temperature was 220°C and the vaporizer tip temperature was varied from 230 to 290°C. The thermospray ion repeller potential was varied from 160 V to 300 V.

Calibration and quality control of the assay

Quantitation of the alkaloids was based on the internal standard method. Five-point calibration curves (triplicate injections) were created for the range from 250 pg to 250 ng per injection by plotting the ratios of analyte and internal standard (40 ng of homatropine) *vs.* the amounts of analyte. Linear regression was used for calculating the parameters for the curves. Relative standard deviations (R.S.D.) were determined using 8 ng and 250 ng of the alkaloids.

RESULTS AND DISCUSSION

The thermospray mass spectra show abundant protonated molecular ions $(M+H)^+$ for hyoscyamine at *m/z* 290, for scopolamine at *m/z* 304 and for homatropine at *m/z* 276. All the spectra show also an ion at M + 42, presumably $(M+H+CH_3CN)^+$ (Fig. 1). The good sensitivity of alkaloids in the positive-ion thermospray mode can be explained in terms of the ability of the ammonium ion to protonate those solutes, which have high proton affinities [18].

As suggested by Mellon [14], the fragmentation of scopolamine and related alkaloids was obtained by discharge ionization. The fragmentation of scopolamine may be further increased by thermal degradation using a higher vaporizer tip temperature or by collision-induced dissociation with the thermospray ion repeller. The discharge-assisted mass spectra of scopolamine and hyoscyamine show only limited fragmentation at a thermospray capillary temperature of 230°C and an ion repeller potential of 160 V (Figs. 2A and 3A). When the capillary temperature was raised to 290°C the mass spectrum of scopolamine shows fragment ions at the same *m/z* values as the spectrum obtained by methane chemical ionization [19], *e.g.* at *m/z* 286 $(M+H-H_2O)^+$, *m/z* 274 $(M+H-HCHO)^+$, *m/z* 138 (M + H − tropic acid)$^+$, at *m/z* 154 (M + H − tropic aldehyde)$^+$ and at *m/z* 156 (Fig. 2B). The mass spectrum of hyoscyamine shows corresponding fragment

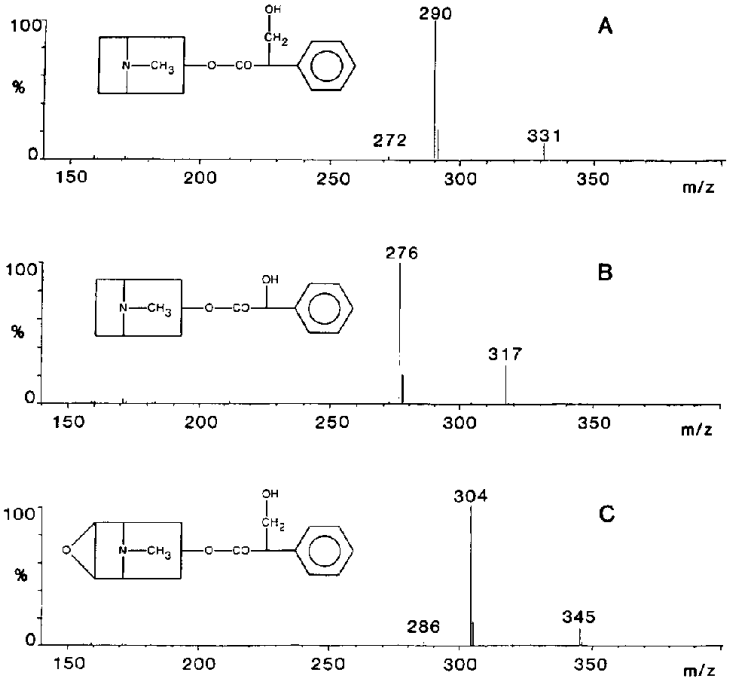

Fig. 1. Molecular structures and thermospray mass spectra of hyoscyamine (A), homatropine (B) and scopolamine (C). The compounds (2 μg) were injected via the column. Conditions: PRP-1 column, 150 × 4.1 mm I.D., 5 μm; mobile phase, 0.1 *M* ammonium acetate–acetonitrile (70:30); flow-rate 1 ml/min; ion source temperature, 150°C; vaporizer tip temperature, 170°C; repeller potential, 150 V; no discharge on and without filament.

ions at *m/z* 272 $(M+H-H_2O)^+$, *m/z* 260 $(M+H-HCHO)^+$, *m/z* 124 $(M+H-$tropic acid$)^+$ and at *m/z* 142 (Fig. 3B). The mass spectra of both compounds show abundant fragment ions formed by loss of tropic acid from the protonated molecular ions when the ion repeller potential is raised from 160 to 300 V (Figs. 2C and 3C).

The new thermospray HPLC–MS method was used for the determination of the hyoscyamine and scopolamine content in plant cell culture samples. Chromatographic separation of the alkaloids was achieved in 6 min with isocratic elution at a flow-rate of 1 ml/min (Fig. 4). The HPLC method was based on a new type of column packing material that allows the use of alkaline eluents. In this case the pH of the eluent was 2.2 pH units higher than the pK_a value of scopolamine (8.2) and 0.7 pH units higher than the pK_a value of hyoscyamine (9.7). The method resulted in relatively good peak shapes and separation of the alkaloids, without the use of alkylsulphonates as ion-pairing reagents.

SIR of the protonated molecular ions was used for quantitation of the compounds. Linear relationships were obtained between analyte to internal standard (homatropine) peak-area ratios and amount of analyte over the range from 250

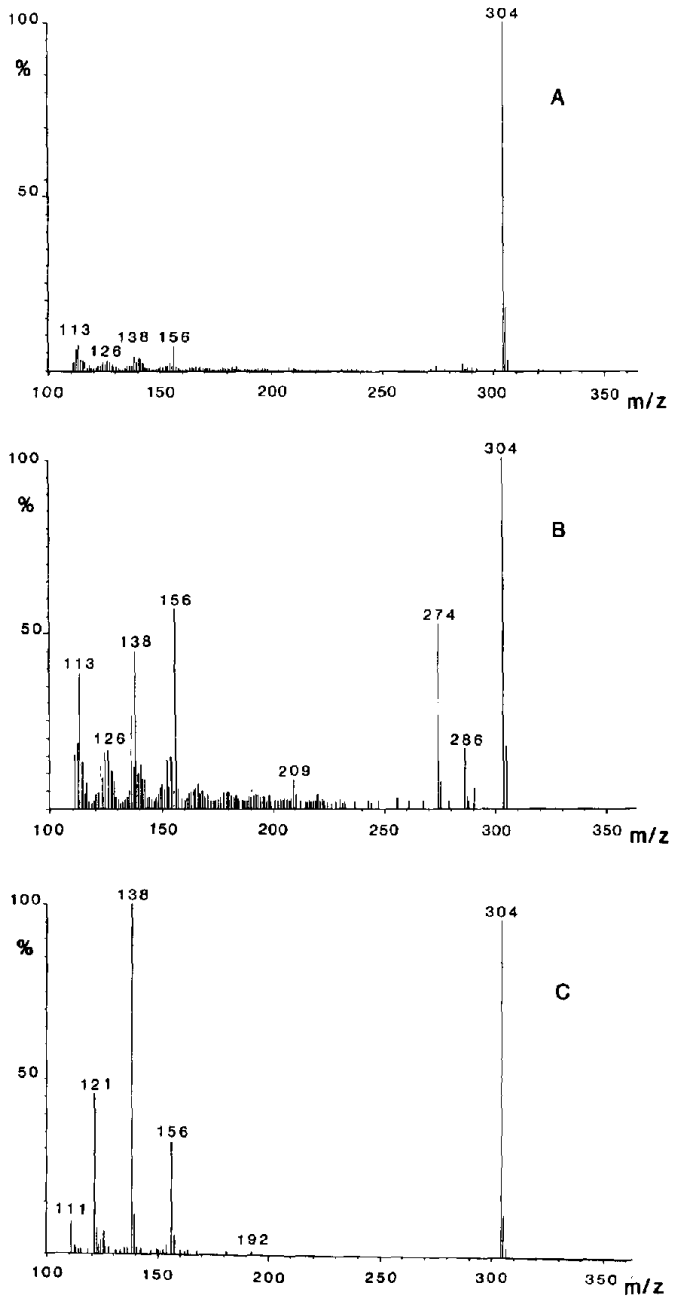

Fig. 2. Discharge-assisted (600 μA) mass spectra of scopolamine obtained with different ion repeller potentials and thermospray vaporizer temperatures. Conditions: (A) vaporizer 230°C, repeller 160 V; (B) vaporizer 290°C, repeller 160 V; (C) vaporizer 230°C, repeller 300 V. The ion source temperature was 220°C and the mobile phase and flow-rate were as in Fig. 1. The compound (20 μg) was injected via a loop.

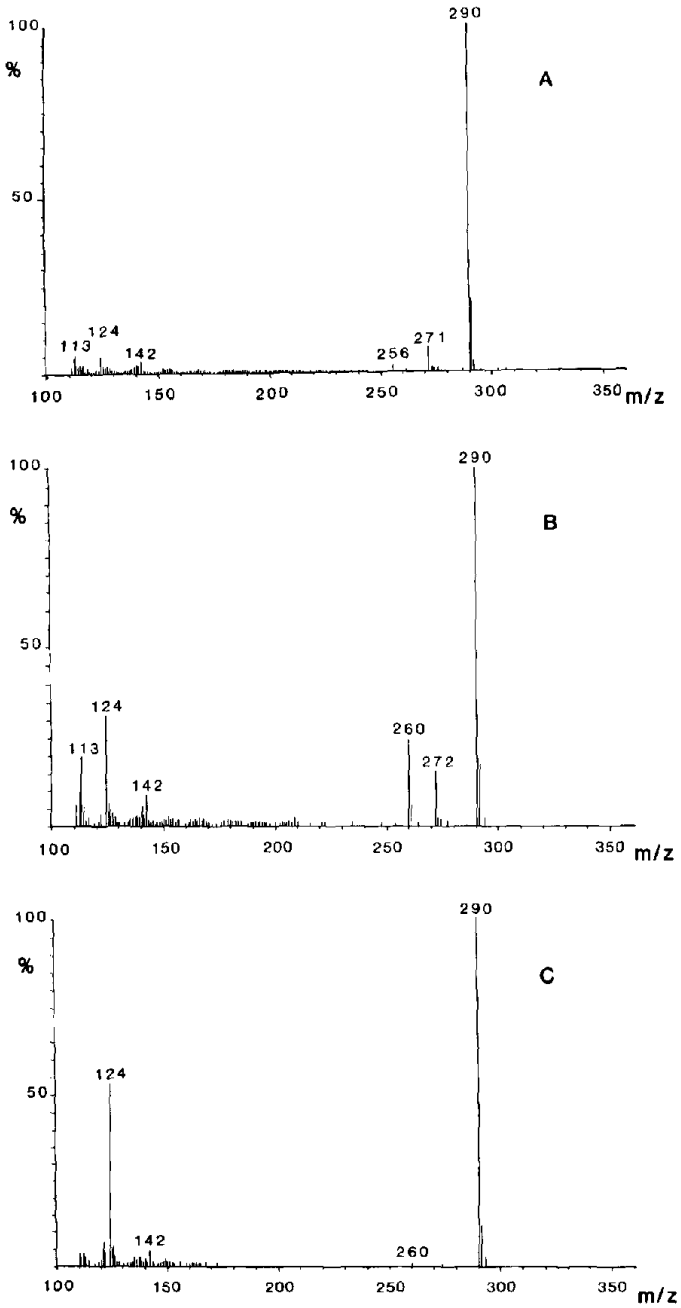

Fig. 3. Discharge-assisted mass spectra of hyoscyamine obtained with different ion repeller potentials and thermospray capillary temperatures. Conditions: (A) vaporizer 230°C, repeller 160 V; (B) vaporizer 290°C, repeller 160 V; (C) vaporizer 230°C, repeller 300 V. Other conditions as in Fig. 2.

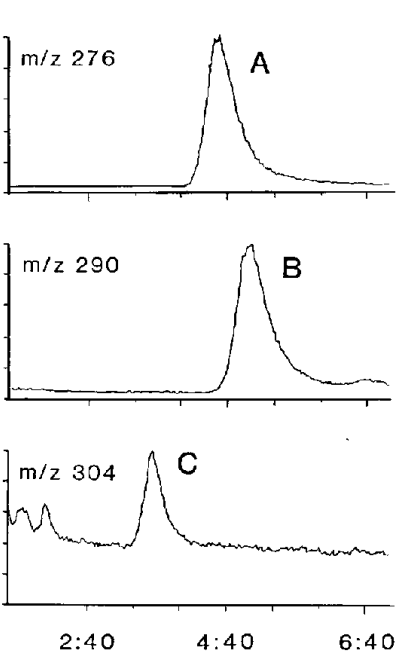

Fig. 4. SIR chromatograms of a root cell culture sample of *Hyoscyamus muticus*. Compounds monitored: homatropine (A) at *m/z* 276 (internal standard), hyoscyamine (B) at *m/z* 290, and scopolamine (C) at *m/z* 304.

pg to 250 ng per injection ($y = 1.688x + 0.0178$, $r = 0.999$ for hyoscyamine, $y = 1.202x - 0.0286$, $r = 0.999$ for scopolamine). The precision was determined for two concentrations, 8 ng ($n = 7$) and 250 ng ($n = 6$) per injection. The R.S.D. were 12.7% (8 ng) and 12.1% (250 ng) for scopolamine, and 17.1% and 3.8% for hyoscyamine. When 50 pg of analyte were injected the signal-to-noise ratio was 10.

This method has potential for the analysis of tropane alkaloids in biological samples. The wide linear range of the assay is useful, because the alkaloid content varies so much between samples from different sources. The discharge-assisted ionization method can be used for the identification of the compounds.

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