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Short Communication

Gas chromatographic analysis of tropic, benzoic and cinnamic acids, biosynthetic tropane alkaloid precursors

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ABSTRACT

A capillary gas chromatographic (GC) method was developed for the determination of tropic, benzoic and cinnamic acids, which are established precursors of many important naturally occurring tropane alkaloids. The acids were derivatized (esterified) by extractive alkylation with pentafluorobenzyl bromide with catalysis by tetrabutylammonium ions. With mandelic acid as internal standard, a direct GC analysis of the methylene chloride extract provides a simple and reliable assay which is applicable to complex sample matrices.

INTRODUCTION

Simple and reliable capillary gas chromatographic (GC) assays for tropic, benzoic and cinnamic acids should prove useful in biochemical research on the medicinally important anticholinergic tropane alkaloids hyoscyamine and scopolamine (tropic acid esters contained, *e.g.*, in *Atropa belladonna* L.) and cocaine (a benzoic acid ester occurring as the main tropane alkaloid constituent of *Erythroxylon coca* Lam.) [1–4]. The acids in question have been assayed both by high-performance liquid chromatography (HPLC) and by GC [5–8]. A promising method for the assay of carboxylic acids seems to be that described by Greving *et al.* [9]. A reliable GC method has been described for the quantification of some of the most important tropane alkaloid precursor amines potentially occurring in *A. belladonna* cell cultures [10].

This investigation was aimed first at the development of a reliable tropic acid assay on the basis of phase-transfer catalysis ("extractive alkylation") and capillary GC [9]. Combination of extraction and derivatization steps is advantageous for the determination of many compounds with exchangeable protons, procedural simplicity providing improved analytical reliability [9,11,12]. From an analytical point of view, the primary alcoholic hydroxyl group of tropic acid renders it more difficult to determine in comparison with benzoic and cinnamic acids (see Fig. 1 for structures),

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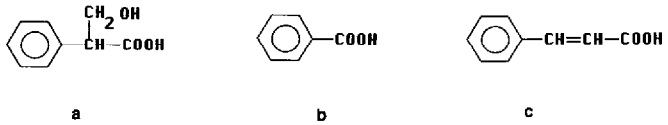

Fig. 1. Structures of (a) tropic, (b) benzoic and (c) cinnamic acid.

the last two compounds being included here as analytes for potential future applications.

EXPERIMENTAL

A stock solution containing 1 mg of each of tropic acid (EGA-Chemie), benzoic acid (Merck) and cinnamic acid (Merck) in 1 ml of methanol was used for the preparation of standard samples, which were obtained by mixing aliquots of the above solution with lyophilized cells (50 mg) from an *A. belladonna* cell suspension culture devoid of the acids in question. The tetrabutylammonium hydroxide (TBA-OH) counter-ion solution (0.1 *M*, pH 8) for phase-transfer catalysis was obtained from TBA-HSO_4 (Fluka) by neutralization with 2 *M* NaOH solution and dilution with phosphate buffer solution (pH 8) (0.050 *M* KH_2PO_4–0.045 *M* NaOH). The alkylating agent, pentafluorobenzyl bromide (PFB-Br) (EGA-Chemie), was used as a 0.5% solution in methylene chloride.

Lyophilized cell samples (50–100 mg) were shaken in test-tubes with 1 ml of buffer solution (pH 8). Following the addition of 1 ml of the counter-ion solution, 2 ml of PFB-Br solution and 75 μl of an internal standard solution containing mandelic acid (Merck) in methanol (1 mg/ml), the tubes were first shaken for 30 min at room temperature and then placed in an ultrasonic bath (45°C) for 30 min. After centrifugation, the aqueous upper layer was withdrawn by suction. The dried (addition of Na_2SO_4 followed by centrifugation) methylene chloride solution (1 μl) was used for GC analysis.

The alkylation procedure was tested with tropic acid at five pH values (6, 7, 8, 9 and 10; phosphate buffer) plotting the resulting PFB ester peak areas against pH.

To test the hydrolytic stability of hyoscyamine and scopolamine in the alkylation process, 1 m*M* solutions were added to *A. belladonna* cell suspension samples. Even for 4 h shaking/ultrasonic treatments (see above), the chromatograms confirmed the total absence of tropic acid PFB ester peaks.

The GC analysis was carried out with a 25 m × 0.32 mm I.D. OV-1701 fused-silica capillary column with a 0.25-μm coating (Nordibond, Nordion) in a Dani HR 3800 gas chromatograph. The instrument was equipped with a flame ionization detector and a programmed-temperature vaporizer (PTV). Hydrogen was used as the carrier gas at a flow-rate of 3 ml/min and the temperature was programmed from 80 to 255°C at 12°C/min. The PTV temperature range was 70–250°C (splitless operation).

RESULTS AND DISCUSSION

Phase-transfer catalysed alkylation provides an excellent analytical approach for many polar active hydrogen compounds. A wide range of commercially available

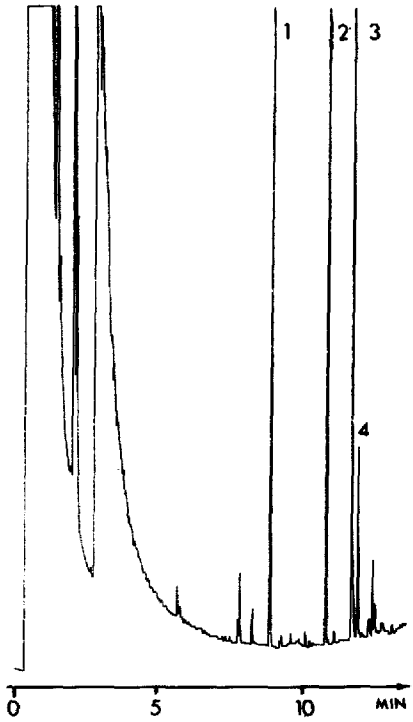

Fig. 2. Gas chromatogram of a standard sample. Peaks: 1 = benzoic acid; 2 = mandelic acid (internal standard); 3 = cinnamic acid; 4 = tropic acid (as PFB esters).

alkylating agents allows the attainment of a sufficient degree of lipophilicity to yield rapid and quantitative extractions with relatively non-polar organic solvents, the derivatization of phenolic compounds, carboxylic acids, barbiturates, etc., thus being easily accomplished. Extraction is based on ion-pair formation, the anionic component of the ion pair being highly susceptible towards derivatization by a suitable carbocation source in organic solution. Even moderately polar compounds are rapidly and quantitatively derivatized, their anions being continuously removed from the aqueous phase by the alkylation process. The aqueous phase pH must be adjusted to allow for the presence of a reasonable ion-pair fraction in the organic phase. The high alkylating power of PFB-Br stems from a good relative stability of the carbocation arising from carbon–bromine bond cleavage. In many GC applications this alkylating reagent has been successfully used for direct drug alkylations within biological sample matrices [9,11,12].

Currently the most advanced tropic acid assays seem to be those based on HPLC [5–7], although in comparison with HPLC methods the present GC assay should provide better separations. In most GC applications an extraction procedure is followed by silylation [13–15]. At least one extraction step can be avoided and the extractant volume can be essentially reduced in the present method, which allows the simultaneous separation and determination of all acids of interest, as demonstrated by the chromatogram of a standard sample in Fig. 2.

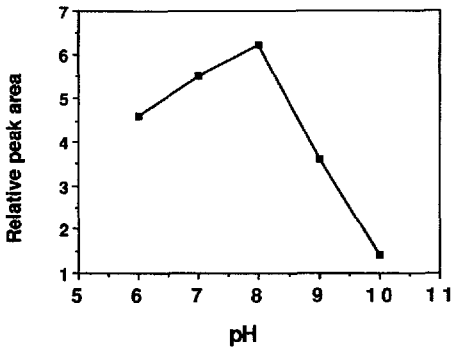

Fig. 3. pH dependence of tropic acid PFB ester peak area in the chromatograms.

Relative simplicity of sample treatment is the outstanding feature of the method, the reaction conditions also being sufficiently mild to leave ester bonds intact (*cf.*, Experimental). It is noteworthy that hydrolytic cleavage of ester alkaloid structures could theoretically provide a source of analytical error through potential "PFB transesterification". pH 8 was found to be optimum for the PFB alkylation of tropic acid (Fig. 3).

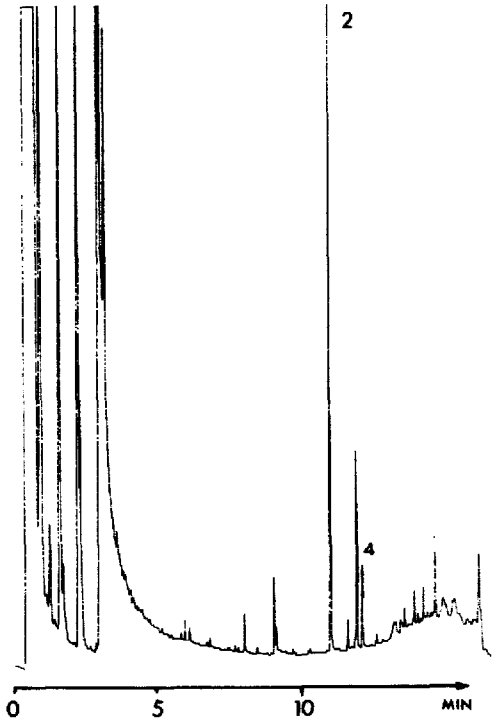

Fig. 4. Gas chromatogram of a cell suspension sample of *Atropa belladonna* [16]. Peaks: 2 = mandelic acid (internal standard); 4 = tropic acid (as PFB esters).

TABLE I

ANALYTICAL PARAMETERS OF THE ASSAY FOR TROPIC, BENZOIC AND CINNAMIC ACIDS

Parameter	Tropic acid	Benzoic acid	Cinnamic acid
Equation of calibration graph[a]	$y = 0.94x - 0.018$	$y = 5.71x - 0.099$	$y = 2.62x - 0.003$
Correlation coefficient (R)	0.995	0.998	0.999
Relative standard deviation ($N = 9$, 30 μg/ml)	5.4	3.5	3.6
Detection limit (ng injected)	5	2	2

[a] y = Peak area ratio; x = concentration (μg/ml) × 10^{-2}.

Table I lists the analytical parameters obtained for the compounds of interest. Of course, a considerable increase in sensitivity can be obtained in GC, if necessary, by the use of an electron-capture detector. The separation of tropic acid PFB ester from various co-analytes present in a suspension culture sample of *A. belladonna* is shown in Fig. 4. The average tropic acid recovery was 113.1% and 97.5% at 10.0 and 40.0 μg, respectively, from spiked cell suspension samples (100 mg, $n = 5$).

The method has been successfully applied in alkaloid and alkaloid precursor feeding experiments involving *A. belladonna* cell suspension cultures [16].

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