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HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF CANNABIS

QUANTITATIVE ANALYSIS OF ACIDIC AND NEUTRAL CANNABINOIDS

R. N. SMITH and C. G. VAUGHAN

Metropolitan Police Forensic Science Laboratory, 109, Lambeth Road, London SE1 7LP (Great Britain)

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SUMMARY

A reversed-phase high-pressure liquid chromatographic method has been developed for the simultaneous analysis of the acidic and neutral cannabinoids in cannabis. Cannabigerol and cannabigerolic acid have been located in the liquid chromatogram of cannabis and factors affecting the chromatographic process are discussed. A method for quantitating one component in the presence of a second unresolved component is described.

INTRODUCTION

A reversed-phase high-pressure liquid chromatographic (HPLC) method suitable for routine comparative cannabis analysis has been described[1]. A subsequent paper[2] reported the identification of a number of cannabis constituents (cannabinoids) that could be separated by HPLC. The excellent separation of neutral and acidic cannabinoids indicated that HPLC would be a useful method for the quantitative analysis of cannabinoids, and this paper describes the development of such a method and its application to cannabis analysis.

EXPERIMENTAL

AnalaR solvents were used throughout.

High-pressure liquid chromatography

The chromatographic system was that described previously[1,2]. The reducing union at the bottom of the column was fitted with a disk of glass-fibre paper sandwiched between two disks of 8 μ stainless-steel gauze. A disk of 8 μ stainless-steel gauze was laid on top of the column and held in place by an oversized disk of 400-mesh stainless-steel gauze that was crimped down with the end of a steel rod to form a cup-shaped insert. The column was lagged and heated electrically to 26 $\pm$ 0.5° so that constant retention times were obtained. Two Cecil 212 variable-wavelength UV

detectors were connected in series so that the eluant could be monitored at 220 and 254 nm sequentially.

Samples of 100 mg of finely ground cannabis resin were extracted for 10 min in an ultrasonic bath with 1 ml aliquots of chloroform–methanol (1:9) containing 8 g/l of di-*n*-octyl phthalate (Phase Separations, Queensferry, Great Britain) as the internal standard. Herbal cannabis (*ca.* 200 mg samples) or cannabis extracts ("hash oils"; *ca.* 10 mg samples) were extracted similarly. The extracts were centrifuged and 2 μl aliquots were injected on to the top of the HPLC column with the end of the needle touching the stainless-steel gauze. With a flow-rate of 2 ml/min at *ca.* 2750 p.s.i., a typical chromatogram took about 25 min to run. Extracts were stable for up to 72 h in the dark at −18°, but slight decomposition occurred at room temperature in daylight during an 8-h period.

Gas–liquid chromatography

A 2 m × 0.4 cm glass column packed with 3% OV-17 on 100–120 mesh Gas-Chrom Q, a nitrogen carrier gas flow-rate of 60 ml/min, an oven temperature of 240°, and a flame ionisation detector were used.

Cannabis samples were extracted as described above, and the extracts were diluted fivefold with methanol for analysis by GLC. The di-*n*-octyl phthalate in the extraction solvent served as the internal standard.

Location of cannabigerol and cannabigerolic acid on HPLC

Cannabigerol (CBG) and cannabigerolic acid (CBGA) were located on HPLC (Fig. 1) using methods previously described[2]. The identity of CBG was confirmed by gas chromatography-mass spectrometry and the mass spectrum agreed well with published data[3].

Calibration

Peak heights relative to the internal standard were used for calibration on both GLC and HPLC.

On HPLC, Δ^9-tetrahydrocannabinol (THC), cannabidiol (CBD), and cannabinol (CBN) were calibrated at 220 nm, while the corresponding acidic cannabinoids Δ^9-tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA), and cannabinolic acid (CBNA) were calibrated at 254 nm. Prior conditioning of the HPLC column by running a number of samples ensured stable calibrations and the graphs were linear over the required ranges. Calibrations for CBG, CBGA, cannabichromene (CBCh), and cannabichromenic acid (CBChA) were not obtained since samples of known concentration were not available.

HPLC and GLC calibrations for THC, CBD, and CBN were obtained by running solutions of pure standards in the extraction solvent–internal standard mixture.

The acidic cannabinoids, for which no standards were available, were quantitated by GLC and the data obtained were used for the HPLC calibration. This was done by extracting the acidic cannabinoids from a cannabis resin sample, separating them into two fractions by HPLC[2] and examining these by GLC. Decarboxylation of the acids by the heat of the injection port[4] gave the corresponding neutral cannabinoids which were quantitated by reference to the GLC calibration graphs. The

concentrations of the acidic cannabinoids in the two fractions were then calculated (assuming 100% decarboxylation) and suitable dilutions of the fractions were used to obtain the HPLC calibration. One acid fraction contained CBDA and CBGA, while the other contained THCA, CBNA, and CBChA. Fractionation of the acids was necessary to avoid interference between CBD and CBCh on GLC[5].

QUANTITATION OF UNRESOLVED COMPONENTS

On HPLC at ambient temperature, CBG appears as a trailing shoulder on the CBD peak, and CBGA and CBN are unresolved. CBG, however, is a minor constituent of cannabis compared with CBD and the absorption spectra and extinction coefficients of the two compounds are similar, so in most cases CBG will have a minimal effect on the CBD quantitation. The effect of CBGA on the CBN quantitation is more serious and cannot be ignored, but the interference can be eliminated by (a) raising the column temperature to resolve CBGA and CBN or (b) calculation.

By running the HPLC column at *ca.* 40°, CBGA and CBN are sufficiently resolved (Fig. 1C) for CBN to be quantitated (but the resolution of CBG and CBD is not improved). The higher temperature introduces no new problems; the column efficiency is increased, the retention times are decreased and, if extreme accuracy is not required, the calibrations obtained at 26° may be used since they are valid to within a few per cent at 40°.

An alternative is to run the column at 26° and quantitate CBN in the unresolved CBGA–CBN peak by calculation, for peak height measurements at the two wavelengths provide sufficient information to do this without having to calibrate for CBGA, although a sample of CBGA unmixed with CBN is required.

For two unresolved components, A and B, the basic relationships are

$$R_1 = \frac{H_1}{S_1} = \frac{C_A}{F_{1A}} + \frac{C_B}{F_{1B}} \tag{1}$$

and

$$R_2 = \frac{H_2}{S_2} = \frac{C_A}{F_{2A}} + \frac{C_B}{F_{2B}} \tag{2}$$

where H_1 and H_2 are the peak heights of the mixture of A and B at wavelengths 1 and 2; S_1 and S_2 are the internal standard peak heights at wavelengths 1 and 2; C_A and C_B are the concentrations of A and B, and F_{1A}, F_{2A}, F_{1B} and F_{2B} are the calibration factors for A and B at wavelengths 1 and 2, *i.e.*,

$$F_{1A} = C_A \text{ when } \frac{\text{Peak height of A at wavelength 1}}{\text{Internal standard peak height at wavelength 1}} = 1$$

From eqns. 1 and 2

$$F_{1B} \cdot R_1 = \frac{C_A \cdot F_{1B}}{F_{1A}} + C_B \tag{3}$$

and

$$F_{2B} \cdot R_2 = \frac{C_A \cdot F_{2B}}{F_{2A}} + C_B \tag{4}$$

Subtracting eqn. 4 from eqn. 3 to eliminate C_B gives

$$F_{1B} \cdot R_1 - F_{2B} \cdot R_2 = C_A \left[\frac{F_{1B}}{F_{1A}} - \frac{F_{2B}}{F_{2A}} \right] \tag{5}$$

therefore

$$C_A = \frac{\frac{F_{1B}}{F_{2B}} \cdot R_1 - R_2}{\frac{F_{1B}}{F_{2B}} \cdot \frac{1}{F_{1A}} - \frac{1}{F_{2A}}} \tag{6}$$

In practice, R_1 and R_2 are calculated from peak height measurements and F_{1A} and F_{2A} are found from the calibration curves. The ratio F_{1B}/F_{2B} is found by running a pure sample of B with the internal standard at wavelengths 1 and 2. Since the concentration of B is the same at both wavelengths

$$\frac{H_{1B}}{S_1} \times F_{1B} = \frac{H_{2B}}{S_2} \times F_{2B}$$

where H_{1B} and H_{2B} are the peak heights of B at wavelengths 1 and 2 and S_1 and S_2 are the internal standard peak heights at wavelengths 1 and 2; therefore

$$\frac{F_{1B}}{F_{2B}} = \frac{\frac{H_{2B}}{S_2}}{\frac{H_{1B}}{S_1}}$$

Thus F_{1B}/F_{2B} is easily calculated from peak height measurements and values of C_A can be obtained from eqn. 6 without having to calibrate for B. It is assumed that A and B are exactly coincident in the chromatogram, and obviously the formula is inapplicable if the extinction coefficients of A and B change by the same relative amount on changing the detector wavelength, for then A and B would be optically indistinguishable.

Errors are minimised if the peaks are reasonably high at both wavelengths and, assuming no errors in F_{1A}, F_{2A}, and F_{1B}/F_{2B}, the variance of C_A, $\sigma^2_{C_A}$, is given by

$$\sigma^2_{C_A} = \frac{\left[\frac{F_{1B}}{F_{2B}}\right]^2 \cdot \sigma^2_{R_1} + \sigma^2_{R_2}}{\left[\frac{F_{1B}}{F_{2B}} \cdot \frac{1}{F_{1A}} - \frac{1}{F_{2A}}\right]^2} \tag{7}$$

where $\sigma^2_{R_1}$ and $\sigma^2_{R_1}$ are the variances of R_1 and R_2.

Results for the quantitation of CBN in cannabis resin using the method are given in Table I. For the 23 analyses of the same extract, the standard deviation (6.8% of the mean) calculated from the individual results reflects the additional variables employed in the calculation and agrees with the value obtained (6% of the mean) using eqn. 7. The standard deviation of 3.3% of the mean obtained when ten samples of a different resin were analysed is probably fortuitously small.

TABLE I

MEAN CANNABINOID LEVELS GIVEN AS PERCENTAGE BY WEIGHT AND (IN PARENTHESES) STANDARD DEVIATIONS OF RESULTS EXPRESSED AS PERCENTAGE OF MEAN

*Cannabinoid**	*23 analyses of a single extract of cannabis resin*	*10 samples of a similar resin***
THC	3.86 (2.1)	2.84 (2.4)
CBD	0.67 (1.2)	0.62 (1.3)
CBN****	0.35 (6.8)	0.54 (3.3)
THCA	4.76 (1.9)	4.20 (2.3)
CBDA	1.54 (2.0)	1.38 (2.3)
CBNA	0.35 (1.7)	0.35 (1.8)

* Abbreviations are explained in the text.

** Each sample was chromatographed twice and the averages of the duplicates were used to calculate the means and standard deviations.

*** By calculation from unresolved CBN–CBGA peak as explained in the text.

When the cannabis extract used for the 23 replicate analyses was run at 36.5°, CBN and CBGA were partially resolved and duplicate determinations of CBN by reference to the calibration graph gave results of 0.35 and 0.36%. In addition, when eqn. 6 was applied to pure CBN or pure CBGA, the results agreed with those expected to well within the experimental error.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatograms of a typical sample of cannabis resin. Table I gives the means and standard deviations of the cannabinoid levels in cannabis resin when (a) the same extract of a resin was analysed 23 times, and (b) ten samples of a similar resin were each analysed twice.

There are obvious practical advantages in measuring both acidic and neutral cannabinoids on a single analytical system at a temperature only slightly above ambient. GLC has been used extensively for cannabinoid quantitation[5], but cannabivarin (CBNV), CBD, and CBCh are not resolved on an OV-17 column, and on OV-1 or OV-101, although CBNV, CBD and CBCh are resolved, CBN and CBG are not and they also coincide with a C_{29}-hydrocarbon that occurs in cannabis and with androst-4-ene-3,17-dione, which is used as an internal standard. In addition, acidic cannabinoids cannot be run on GLC without preliminary derivatisation, and this can cause problems in quantitative analysis if the reaction is incomplete or if silyl derivatives contaminate the detector.

Most of these difficulties can be avoided by using HPLC, though interference due to minor cannabis constituents such as the propyl cannabinoids, *e.g.*, CBNV, has not been investigated. HPLC offers considerable potential, not only in forensic work but in pharmacological and chemobotanic studies where it is necessary to know the detailed cannabinoid content of the material used. If only the THC and THCA (or CBD and CBDA) levels are of interest, the analysis can be carried out using a single detector at 220 nm since THCA (and CBDA), unlike the other acids, absorb strongly at this wavelength.

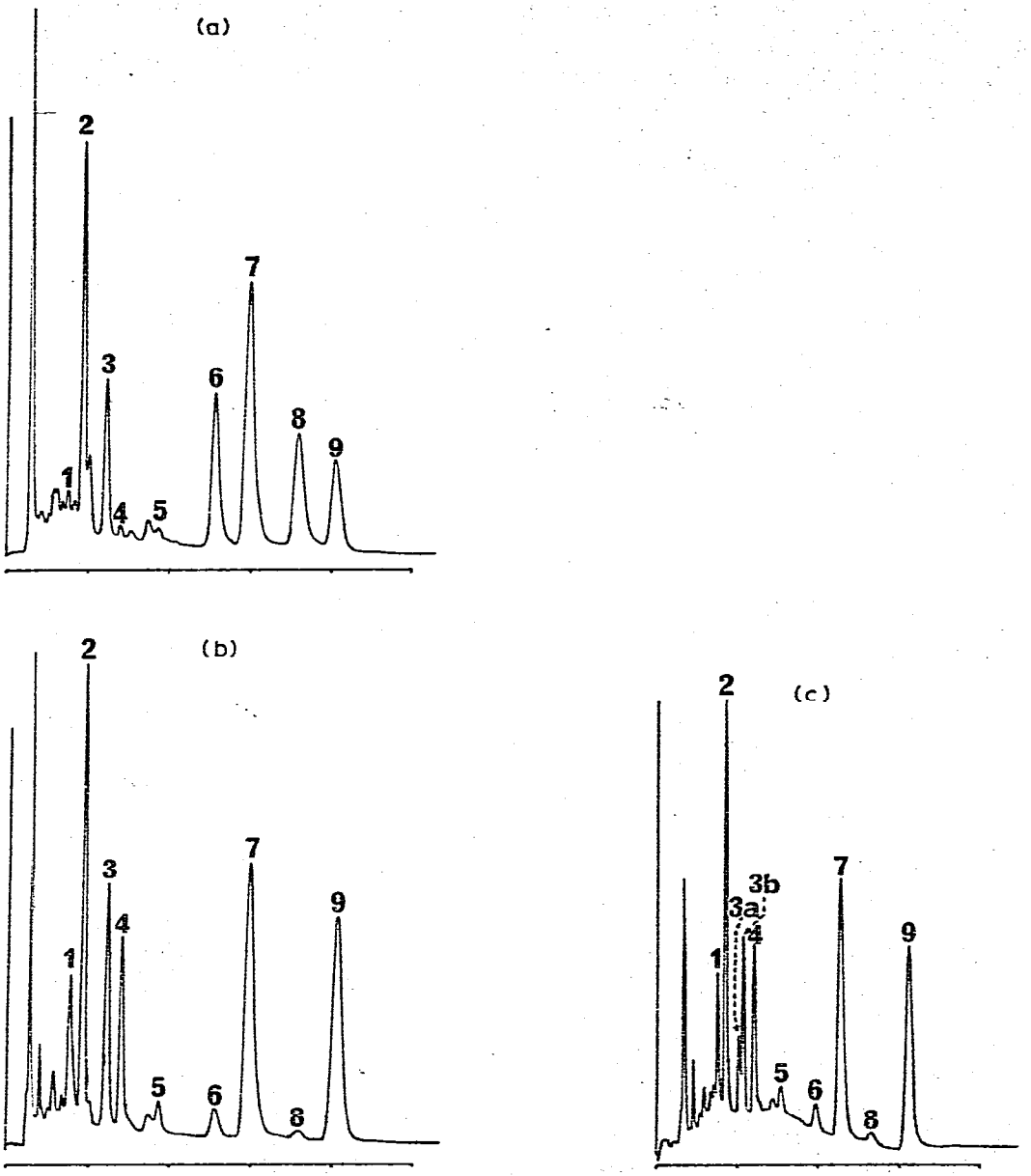

Fig. 1. HPLC of cannabis resin at (a) 254 nm and 26°, (b) 220 nm and 26°, and (c) 220 nm and 36.5° (1.25-μl injection). Chromatographic conditions: 100 mg resin extracted with 1 ml chloroform–methanol (1:9) containing 8 g/l di-*n*-octyl phthalate; 2 μl extract injected (stop-flow injection) on to 25 cm × 4.9 mm I.D. stainless-steel column packed with silica-C_{18} (medium load of C_{18} on Partisil 5); eluting solvent, methanol–0.02 *N* H_2SO_4 (4:1); flow-rate, 2 ml/min at *ca.* 2750 p.s.i.; detectors, Cecil 212 variable-wavelength; absorbance, 0.2 at 254 nm and 0.5 at 220 nm. Scale graduations represent 5-min intervals. Identity of peaks (abbreviations are explained in the text): 1 = CBD and CBG (shoulder); 2 = CBDA; 3 = CBN and CBGA; 3a = CBGA; 3b = CBN; 4 = THC; 5 = CBCh; 6 = CBNA; 7 = THCA; 8 = CBChA; 9 = di-*n*-octyl phthalate (internal standard).

The choice of internal standard was an important step in the development of an HPLC method for quantitative cannabis analysis, for it had to have appropriate UV extinctions at 220 and 254 nm as well as a suitable retention time. Numerous compounds were tested, but only di-*n*-octyl phthalate and di-*n*-nonyl phthalate satisfied the necessary criteria. Of the two, di-*n*-octyl phthalate had the better retention time and it had the additional advantage of being a suitable internal standard for GLC as well as HPLC. Diisooctyl phthalate, which is sometimes advertised as dioctyl phthalate in chemical catalogues, is unsuitable as an internal standard.

Initially the internal standard was dissolved in eluting solvent (methanol–0.02 *N* H_2SO_4, 4:1) and this solution was used to extract the cannabis samples. However, insufficient internal standard dissolved in the eluting solvent to give a reasonable peak height, and the larger injections that were then necessary gave poor peak shapes. In addition, the extractions were incomplete although they were reproducible, and the internal standard was partially absorbed by particulate matter in the cannabis samples. These problems were avoided by dissolving the internal standard in a suitable organic solvent and using this to extract the cannabis samples. The choice of solvent is important. Methanol has been found to cause loss of resolution when it is used as the injection solvent in cannabis HPLC, and it was suspected that this was due to partial precipitation of the sample on top of the column[1]. This has been re-investigated in the present study using an injection port attached to a short glass column. No precipitation was observed, but the methanol solution was seen to stream upwards into the dead-space in the injection port. This would result in an inefficient injection, and accumulation of the sample in the injection port could lead to loss of resolution if diffusion of sample into the mobile phase occurred during analysis. Chloroform–methanol (1:9) was found to be a suitable injection solvent since (a) it is slightly denser than the eluting solvent, (b) the internal standard is easily soluble in it, (c) it extracts cannabinoids quantitatively from cannabis, and (d) absorption of internal standard by particulate matter in the samples does not occur.

The need for a "balanced-density injection" obviously depends on the geometry of the injection system, but the density of the injection solvent is not the sole criterion for long-term reproducibility in reversed-phase HPLC. We obtained reproducible chromatograms from 2-μl injections using the chloroform–methanol (1:9) solvent, but 5-μl injections gave less reproducible results and preparative-scale injections of 50–100 μl led to a rapid loss of column efficiency. Solvent washes or repacking the top of the column did little to restore the efficiency; this could only be done by repacking the whole column.

It has been suggested[6] that polar organic solvents injected on to a reversed-phase HPLC column displace water molecules from hydrated residual silanol groups, generating active sites that can retain solutes and cause tailing peaks. This hypothesis does not, however, account for the permanent loss of efficiency that we observed with large injections. It is likely that several factors are involved and no explanation can be put forward at present. It may be relevant to note that pre-conditioning of some columns by eluting with 500 ml methanol–0.1 *N* H_2SO_4 (4:1) reduced tailing and improved the peak shapes.

Despite the variables that can affect the results of reversed-phase HPLC, no long-term stability problems have been encountered in the analysis of a wide range of cannabis samples using the method described in this paper.

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