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Short Communication

Determination of ^{14}C-THC and ^{14}C-11-OH-THC from *in vitro* metabolism studies by mass spectrometry (GC–MS) or on-line radiometric detection (HPLC-R)*

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Introduction

The detection of delta-9-tetrahydrocannabinol (THC) and its biotransformation products in studies related to its metabolism has been described using GC–MS methods [1, 2] due to the high sensitivity needed in the detection of the small amounts of metabolites formed. The alternative use of a radioactive substrate had already been described in initial works, separation being performed by TLC [3] or paper chromatography [4] and detection by liquid scintillation counting of the separated fractions. The promising use of high-performance liquid chromatography (HPLC) for the separation and detection of THC labelled metabolites has not been described. In this work, on-line radiometric detection after reversed-phase HPLC separation (HPLC-R) has been developed and the results compared with gas chromatography–mass spectrometry (GC–MS). This method is being used to study the metabolism of ^{14}C-THC by human hepatic and pulmonary tissue and its possible induction or inhibition by several compounds.

Experimental

Apparatus

In HPLC-R, the following instruments were used: a Perkin–Elmer model ISS 100 automatic liquid sampler, a Hewlett Packard (HP) 1050 series pumping system, a Beckman 165 variable wavelength detector, a Beckman 110B solvent delivery module for the scintillation cocktail, and a Beckman 171 radioisotope detector. In the efficiency study, a LKB 1214 Rackbeta liquid scintillation counter was used. Data were integrated by the Beckman PC software Library — Data Capture Module Rev. 2.3 software.

In GC–MS, the instruments used were: an HP 5890A gas chromatograph with a 7673A HP automatic sampler coupled to an HP 5970A mass selective detector. Data were processed with the HP 5970 MS ChemStation software.

Samples, chemicals and standards

The human liver microsomes used were a gift from the Cost Action B1 European Programme [5]. The rat liver microsomes were prepared in the Department as previously described [6]. The substrate (11-^{14}C-THC, specific activity 0.157 mCi mg^{-1}), the internal standard used in GC–MS (5′,5′,5′-$^{2}H_3$-THC) and the reference product 11-OH-THC were a gift from the Research Triangle Institute (USA). These substances were dissolved in ethanol and stored at −20°C. For the prep-

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aration of the mobile phase, gradient grade reagents were used. Extraction solvents were analytical grade. The scintillation cocktail used in HPLC-R with liquid cell detection was Beckman Ready Flow III (P/N 158734).

Analytical techniques

In HPLC-R, a pre-column (Waters RCSS silica guard-pack) was connected to a Beckman ultrasphere 5 μm, 4.6 mm × 25 cm analytical column. The mobile phase was a mixture of acetonitrile–methanol–water (80:10:10, v/v/v) and the flow rate was 0.7 ml min^{-1}. The scintillation cocktail flow rate was 3 ml min^{-1}.

In GC–MS, the column was a HP cross-linked methylsilicone gum, 25 m × 0.2 mm × 0.11 μm film thickness. The initial temperature of the oven was 100°C for 1 min, and an increase rate of 20°C min^{-1} was applied until the temperature rose to 300°C. This was maintained for 3.5 min. The acquisition mode was single ion monitoring (SIM). The ions monitored for each compound were: for ^{14}C-THC, *m/z* 303, 315, 343, 371 and 388; for $^{2}H_3$-THC, *m/z* 374 and 389; and for ^{14}C-OH-THC, *m/z* 207, 303, 369, 371, 403, 459 and 476.

Incubation procedure

The substrate solution (500 ng ^{14}C-THC (0.08 μCi)) was added to each tube, except to the control blank, and the solvent was evaporated to dryness (ethanol has an inhibitory effect on the enzymic activity of the samples). If inducers or inhibitors are to be used, 100 μl of a 5 mM methanolic solution are added to the tubes and evaporated to dryness. 0.9 ml of the incubation cocktail were added to each tube and mixed in a vortex for 30 s. The incubation cocktail contained, per tube, the following substances: 2.4 mg of magnesium sulphate, 0.5 U isocitric dehydrogenase, 1.29 mg of isocitric acid, and 0.37 mg of NADP in 0.9 ml of phosphate buffer (pH 7.4, 40 mM). Pre-incubation was performed at 37°C for 5 min. After this, 100 μl of microsomes at a concentration of 5 mg ml^{-1} in a solution containing KCl 0.15 M, Tris 50 mM and EDTA 3 mM adjusted to pH 7.4 with HCl were added to each tube, except the blanks. Samples were maintained for 15 min at 37°C and incubation stopped by addition of 10 ml of a mixture of cold hexane–ethylacetate (90:10, v/v). Microsomes were added to the blank tubes, and internal standard (200 ng of $^{2}H_3$-THC) was added to all the tubes, except the control blank. The tubes were shaken in a vortex mixer for 1 min, then for 20 min in a horizontal shaker at 50 movements per min. Samples were centrifuged for 5 min at 2500 rpm. From the organic layer, a 5-ml fraction was separated for the HPLC-R analysis and the remainder used for GC–MS analysis. Both samples were evaporated to dryness under a stream of nitrogen and the fractions for GC–MS analysis kept in a vacuum desiccator for at least 2 h. For HPLC-R analysis, samples were redissolved in 75 μl of mobile phase, and 30 μl injected. For GC–MS analysis, samples were derivatized as previously described [7] and 1 μl injected.

Results and Discussion

Analytical techniques

After HPLC separation, the retention times were 12.6 min for ^{14}C-THC and 7.8 min for ^{14}C-OH-THC. The efficiency of the radioactive detector with a liquid cell (1 ml) in the conditions described for the detection of ^{14}C-THC was 27%. The efficiency of the same method using a solid cell (300 μl) was variable (20–35%) because of its susceptibility to easy contamination. Using a liquid cell, the linearity of the technique to detect ^{14}C-THC, in the range 25–1000 ng (25, 50, 150, 250, 500 and 1000 ng, $n = 3$), was: $Y = 95 + 32X$, $r = 0.9987$, Y being the area in cpms of the peak and X the amount of ^{14}C-THC in ng. When injecting 4 ng of ^{14}C-THC, the signal to noise ratio was 2.1, and 4 ng was considered as the cut-off for detection of ^{14}C-THC. When using a solid cell, the linearity was $Y = -324 + 31X$, $r = 0.9983$ (X and Y being as described for the liquid cell) and the signal to noise ratio was acceptable (2.3) when injecting 8 ng of ^{14}C-THC, this being considered as the cut-off for detection using the solid cell.

In the GC–MS conditions described, ^{14}C-THC and the internal standard ($^{2}H_3$-THC) appeared at 9.33 min and ^{14}C-OH-THC at 10.39 min after CG separation. The linearity of this technique in the same range as described in HPLC-R was, for THC, $Y = 0.0156 + 0.0056X$, $r = 0.9989$; and for OH-THC, $Y = -0.2114 + 0.0102X$, $r = 0.9999$; where Y is the ratio between the area of *m/z* 371 for THC or OH-THC and the area *m/z* 374 for $^{2}H_3$-THC, and X the amount of THC or OH-THC in ng.

When injecting 0.04 ng of THC or OH-THC, the signal to noise ratio of the *m/z* 371

ion was 5.6 for THC and 5.7 for OH-THC, 0.04 ng being considered as the cut-off for detection of THC and OH-THC by the GC–MS technique described. The recovery in the range 50–500 ng ml^{-1} was 84% for THC (n = 6) and 80% for OH-THC (n = 6); this is in spite of previous reports that THC metabolites tend to bind strongly to proteins [8].

Comparing the HPLC-R liquid cell and solid cell techniques, the main difference observed is the limit of detection. It has been reported that the analyte studied has considerable binding affinity for glass, plastic, rubber, etc. [9]. The solid cell scintillator consists of a packing of small particles, giving a large surface area, where ^{14}C-THC could bind causing contamination of the cell. In practice, an increase in the levels of the background of the cell (from 50 to 700 cpms) and a decrease of the counting efficiency (from 35 to 20%) was observed in the solid cell. To analyse routinely compounds with high surface binding affinity, as is the case for the analytes studied in this work, the use of the liquid cell is recommended to avoid the problems described. Using HPLC with UV detection it was not possible to detect the metabolites formed from ^{14}C-THC *in vitro* studies.

Comparing the GC–MS (sim mode) and HPLC-R liquid cell techniques, the cut-off for detection was lower in GC–MS (0.04 ng versus 4 ng) and the peaks were sharper and more easy to integrate. However, the sensitivity of HPLC-R was sufficient to detect the same peaks as in GC–MS in which only ^{14}C-THC and ^{14}C-11-OH-THC (the most abundant metabolite formed [10]) were monitored. By HPLC-R it was possible to detect other unidentified metabolites (retention times 6.3 and 9 min) which appeared as radioactive signals after the incubation. By GC–MS it should in fact be possible to detect all the metabolites formed when working in the scan mode, though then the sensitivity of the technique is decreased. Figures 1 and 2 show representative chromatograms obtained by HPLC-R (liquid cell) and GC–MS.

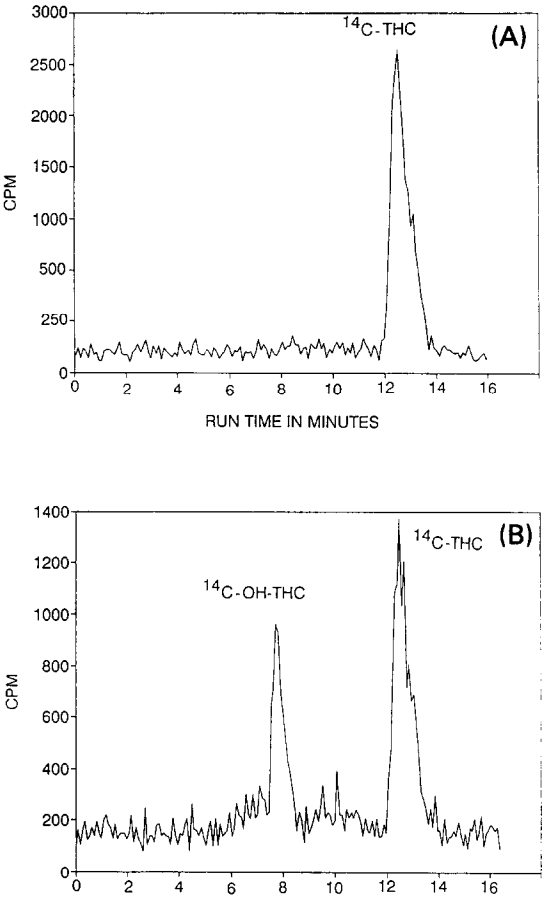

Figure 1
Chromatograms obtained by HPLC-R of: (A) a blank sample, with the microsomes added after the incubation; (B) a problem sample, where the microsomes were added before the incubation and ^{14}C-OH-THC was formed.

Conclusions

HPLC-R appears to be a rapid and inexpensive alternative to GC–MS for ^{14}C-THC metabolic studies. It does not require desiccation or derivatization of the extract.

For the routine analysis of ^{14}C-THC or other ^{14}C labelled compounds with strong surface binding affinity, the use of a liquid rather than a solid cell is recommended.

Using GC–MS it is possible to identify the different metabolites formed during the incubation, but it is necessary to work in the SCAN mode with necessarily decreased sensitivity, or to know previously which ions should be selected for monitoring. Using HPLC-R it is not possible to identify the metabolites, but any significant radioactive signal can be detected. Therefore HPLC-R is a useful screening technique before identification by GC–MS in scan mode when different metabolites are expected.

Both the HPLC-R using a liquid cell and the GC–MS techniques described appear to be suitable for these studies.

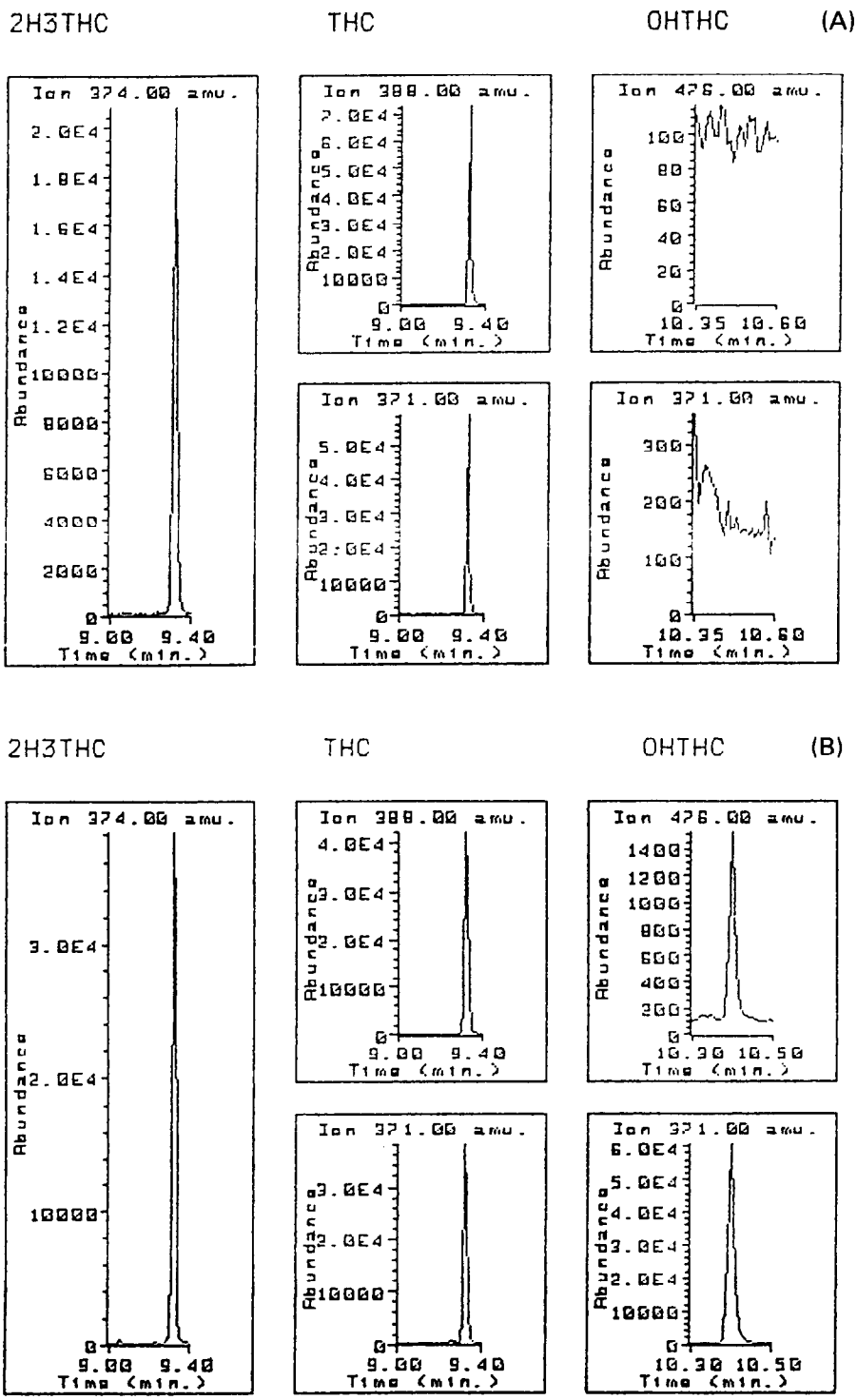

Figure 2
Selective ion chromatograms obtained by GC–MS of: (A) a blank sample with the microsomes added after the incubation. The substrate (^{14}C-THC) was monitored at *m/z* ions 371 and 388. Ion *m/z* 374 corresponds to the internal standard ($^{2}H_3$-THC). (B) A problem sample, where the microsomes were added before the incubation and ^{14}C-OH-THC was formed and monitored for *m/z* 371 and 476.

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