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COMBINED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND RADIOIMMUNOASSAY METHOD FOR THE ANALYSIS OF Δ^9-TETRAHYDROCANNABINOL METABOLITES IN HUMAN URINE

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SUMMARY

A high-performance liquid chromatography–radioimmunoassay method for the measurement of cannabinoids in urine is described. The method involves chromatographing a hydrolysed urine sample with high-performance liquid chromatography and quantifying the eluting cross-reacting cannabinoids with radioimmunoassay. It has been applied to the analysis of cannabinoids in human urine obtained from subjects who had smoked Δ^9-tetrahydrocannabinol and the identities of some of the cross-reacting cannabinoids have been established by gas chromatography–mass spectrometry. The method is suitable for use as a routine procedure for cannabinoid analysis in urine.

INTRODUCTION

Several methods have been reported for the analysis of tetrahydrocannabinol (THC) and its metabolites in plasma. These have employed techniques such as gas chromatography (GC) with flame-photometric detection[1], GC with mass spectrometric (MS) detection[2] and high-performance liquid chromatography (HPLC) with radioimmunoassay (RIA) as the means of detection[3]. The variety of successful methods reported for cannabinoid analysis in urine is, however, not as large.

Thin-layer chromatographic (TLC) procedures for cannabinoid detection in urine have been described[4–7]. These were reviewed by Salaschek *et al.*[8] who concluded that reliable routine detection of cannabinoids in urine was not provided by these TLC methods. A GC–MS method for the quantification of Δ^9-THC-11-oic acid has been described[9], but the application of this procedure to the analysis of this metabolite in urine, obtained after Δ^9-THC administration, was not reported. RIA has been successfully applied to the analysis of cannabinoids in urine[10–12] and this technique

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offers a rapid method for cannabinoid detection in urine although it does not provide the high degree of specificity that might be required for the examination of samples submitted for forensic examination.

The development of further methods for cannabinoid analysis in urine has been hampered by the complexity of the metabolism of THC which is transformed in the body to numerous more polar compounds, some of which have not been identified. *In vivo* and *in vitro* studies in animals have suggested that THC is metabolised via an initial allylic or pentyl side chain hydroxylation. Other reactions may then occur involving the introduction of further hydroxyl groups or oxidation of the introduced hydroxyl group to a carboxylic acid. A final stage may be conjugation with endogenous polar compounds to form more water soluble species[13]. Radiolabelled compounds have been used to investigate the biotransformation of cannabinoids in man and it has been found that the THC metabolites in urine were mainly conjugated polar acids together with smaller amounts of free polar acids such as THC-11-oic acid[14].

A suitable method for cannabinoid detection in urine would, therefore, have to measure low concentrations of polar and possibly conjugated metabolites while avoiding interference from the endogenous material present in urine. The use of a combined HPLC–RIA procedure for the quantification of cannabinoids in plasma has been described previously[3]. HPLC is suitable for the separation of THC and its more polar metabolites and RIA provides a sensitive method for cannabinoid detection which avoids interference from endogenous material present in body fluids. The method has, therefore, now been applied to the analysis of cannabinoids in urine to provide a means of quantifying cross-reacting cannabinoids with high specificity and sensitivity.

EXPERIMENTAL

Collection of samples

Urine containing ^{14}C-labelled Δ^9-THC metabolites was obtained from a rabbit (New Zealand white, 4 kg) 5 h after the injection into an ear vein of 9 μCi (90 μg) of [3′,5′-^{14}C]-Δ^9-THC (Radiochemical Centre, Amersham, Great Britain) which was dissolved in 0.1 ml ethanol and 0.4 ml of saline containing 2% (v/v) Tween 80.

Human urine samples containing Δ^9-THC metabolites were obtained from three volunteers who smoked, over a 10-min period, tobacco cigarettes impregnated with 10 mg (subjects 1 and 2) and 8 mg (subject 3) of Δ^9-THC. Urine samples were collected in silanised glass containers during the 24-h period after smoking and stored at −20° until analysis. Plasma samples were also obtained during this experiment and the analyses of these have been reported (Williams *et al.*[3]).

Control urine samples were obtained from 25 volunteers with no experience of cannabis use.

The source of cannabinoid metabolites used for MS identification was a urine sample received for forensic analysis and found to contain a high level of cannabinoid cross-reacting material (680 ng/ml) in the radioimmunoassay.

Radioimmunoassay

Materials and equipment. Antiserum (133Y/22/5) was obtained from Guildhay

Antisera, University of Surrey, Great Britain. Δ^9-THC and other cannabinoids were generously provided by the National Institute on Drug Abuse, Rockville, Md., U.S.A. and [G-^{3}H]-Δ^9-THC (10 Ci/mmol) was purchased from the Radiochemical Centre.

Method. The RIA procedure used for urine analysis was similar to that described for plasma (Williams *et al.*[3]). A buffered methanol–water (50:50) solution [200 μl, pH 7.4, 0.1 *M* phosphate] of [^{3}H]THC (0.01 μCi) was pipetted into assay tubes together with antiserum (100 μl of 1:300 dilution) and either urine or Δ^9-THC standard. The final assay solution volume was made up to 600 μl with buffer and methanol to give a final methanol concentration of 25%. The solutions were mixed and left to stand at room temperature for 1 h. Dextran coated charcoal (200 μl/tube) was added and after 2 min contact time the tubes were centrifuged. Supernatant (500 μl) from each tube was counted.

High-performance liquid chromatography

Materials and equipment. A Model M-6000A pump (Waters Assoc., Milford, Mass., U.S.A.) was used to deliver methanol–water eluent to a HPLC column (10 cm × 4.6 mm I.D.) packed with Spherisorb-5-ODS (Phase Separations, Queensferry, Great Britain). Samples were introduced onto the column with a Rheodyne Model 905-42 six-port injection valve (Phase Separations) fitted with a 10-ml injection loop. The column eluate was collected in a Struers Samplomat fraction collector (Camlab, Cambridge, Great Britain). A Model SB4 freeze drier (Chemlab Instruments, Ilford, Great Britain) was used to remove solvent. β-Glucuronidase and sulphatase were purchased from Sigma (London, Great Britain).

Method. Methanol and a methanol–water (50:50) buffer (0.1 *M* acetic acid adjusted to pH 5.5 with sodium hydroxide) were added to urine (0.1–3 ml) to give a final volume of 6 ml and a methanol concentration of 50% (v/v). This was injected onto the HPLC column and chromatographed using a stepped solvent elution programme consisting of 10 ml of a mixture of methanol–water (50:50), 10 ml of methanol–water (62.5:37.5) and 20 ml of methanol–water (72.5:27.5) at a flow-rate of 1 ml/min. Eluate fractions were taken every 30 sec. The eluate was monitored for RIA cross-reactivity by removing the HPLC solvent in a freeze drier and assaying the dried residue. The ^{14}C activity was monitored by scintillation counting aliquots of the eluate fractions.

Metabolite conjugates were hydrolysed using alkaline conditions. An equal volume of methanolic sodium hydroxide (1 *M*) was added to the urine, the sample vessel evacuated to remove oxygen and left to stand for 30 min. Sufficient acetic acid was added to give a pH of 5.5. The solution was then diluted to 6 ml with methanol–water (50:50) buffer (pH 5.5) and chromatographed.

Samples of urine were also treated with β-glucuronidase (2000 units/ml), Sigma Type B1) and sulphatase (1000 units/ml, Sigma Type H1) over a 24-h period at 37° and pH 5. Protein was removed from the incubate by the addition of three volumes of methanol followed by centrifugation. The supernatant was prepared for chromatography by the addition of water and methanol–water (50:50) buffer (pH 5.5) to give 6 ml of methanol–water (50:50).

Identification of urinary RIA cross-reacting compounds by GC–MS

Materials and equipment. GC–MS was conducted using a Pye 104 GC equipped

with a 3% OV-17 column (0.3 m × 2 mm I.D., Gas-Chrom Q, 80–100 mesh) interfaced by a jet separator to a VG Micromass 16F mass spectrometer. N,O-bis-trimethylsilyltrifluoroacetamide (BSTFA) with 1% trimethylchlorosilane was purchased from Phase Separations.

Method. Urine (3 ml) was hydrolysed with methanolic sodium hydroxide, chromatographed and eluate fractions taken as described above. Aliquots (5 μl) from each eluate fraction were assayed and those containing RIA cross-reacting material were re-chromatographed using an ODS column (10 cm × 4.6 mm I.D.) with methanol–water (32.5:67.5). Fractions containing cross-reacting material were again identified by assaying aliquots (5 μl) from each eluate fraction and the HPLC solvent was removed by freeze drying.

Cross-reacting compounds were derivatised for GC with BSTFA (10 μl in 30 μl of dry pyridine at 80° for 10 min) and methylated with an ethereal diazomethane solution. Metabolite or metabolite derivatives were gas chromatographed at 220° with a helium flow-rate of 20 ml/min. The mass spectrometer source temperature was 250° and accelerating voltage 4 kV.

TABLE I

LEVELS OF RIA CROSS-REACTION IN URINE SAMPLES OBTAINED FROM VOLUNTEERS WHO HAD SMOKED Δ⁹-THC

Subject	*Time sample was taken after smoking (h)*	*Level of cross-reaction (ng/ml)*	*Urine volume (ml)*	*Total cross-reacting material (μg/h)*
1	Control	35	132	—
	1	117	111	13.0
	2	142	34	4.8
	3	125	56	7.0
	4	145	44	6.4
	6	135	38	2.6
	8	78	113	4.4
	24	49	600	1.8
2	Control	113	95	—
	1	120	92	11.0
	2	67	51	3.4
	3	42	110	4.6
	4	68	66	4.5
	8	15	206	0.8
	24	56	109	0.4
3	Control	0	15	—
	1	3.2	82	0.26
	2	7.3	45	0.33
	3	6.6	50	0.33
	4	3.8	110	0.42
	6	6.2	65	0.20
	8	3.0	166	0.25
	24	3.4	673	0.14

RESULTS AND DISCUSSION

The levels of RIA cross-reaction in urine samples obtained from three subjects who smoked THC impregnated cigarettes are given in Table I. Urine samples from subjects 1 and 2, who were regular cannabis users, gave levels of cross-reaction between 15 and 145 ng/ml over the first 24 h. Levels obtained from subject 3, a non-cannabis user, were lower and were between 3 and 7.3 ng/ml. Control urine samples from subjects 1 and 2 also contained cross-reacting material, probably due to the consumption of cannabinoid material at some time prior to the smoking experiment. Levels of cross-reaction from 25 normal control urine samples were less than 0.5 ng/ml confirming that the RIA is capable of distinguishing normal urine samples from those taken during a period after the smoking of a moderate quantity of Δ^9-THC.

The HPLC retention characteristics of urinary Δ^9-THC metabolites were investigated by chromatographing a sample of rabbit urine containing ^{14}C-labelled metabolites. The column eluate was monitored for radiolabelled compounds by liquid scintillation counting and for cross-reacting compounds with RIA. A comparison of the two chromatograms obtained (Fig. 1) showed that the majority of the metabolites present in the urine did not cross-react in the assay and that there was a major cross-reacting component present with a retention volume of 13.5 ml and a minor component with a retention volume of 16.5 ml. When, instead of the acidic sample pH conditions used here, neutral pH sample conditions were used prior to chromatography, the retention volumes of both of these urinary components decreased to 10 ml. This retention volume is equivalent to that of a major area of cross-reaction observed in the HPLC–RIA of plasma samples (Williams *et al.*[3]). The change in retention volume with pH also suggested that these urinary constituents were acidic.

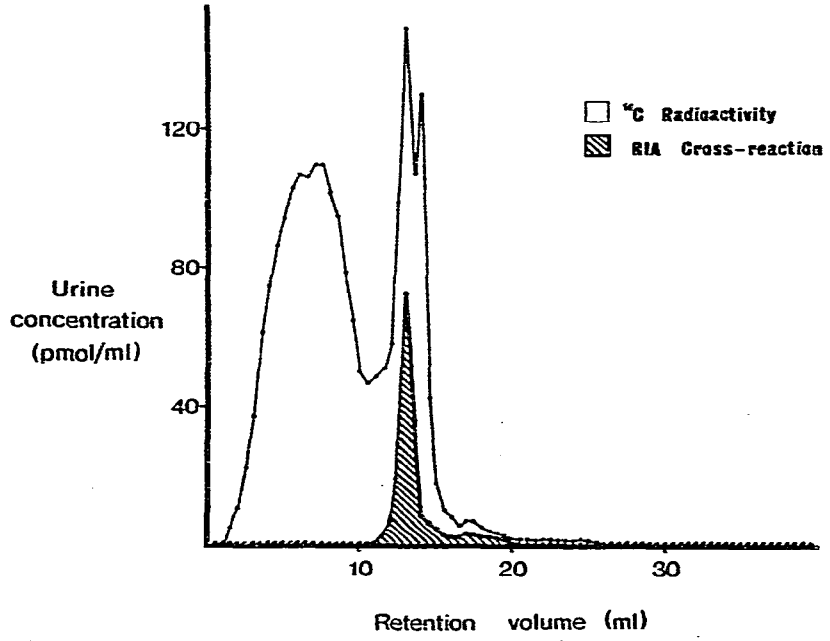

Fig. 1. HPLC elution patterns of radiolabelled Δ^9-THC metabolites and cross-reacting Δ^9-THC metabolites present in an unhydrolysed rabbit urine sample.

When the rabbit urine sample was treated with methanolic sodium hydroxide in an attempt to hydrolyse metabolite conjugates the elution patterns of both cross-reacting and total metabolites changed (Fig. 2). Major cross-reacting components at retention volumes 13.5, 16.5 and 30 ml were now observed as well as an indeterminate number of smaller components. The elution pattern of radioactive metabolites differed from that observed with the unhydrolysed sample by the presence of additional peaks at retention volumes 16.5, 19.5 and 30 ml. The component at retention volume 19.5 ml did not cross-react in the assay unlike the components at retention volumes 16.5 and 30 ml which gave equivalent quantitative results by both RIA and ^{14}C measurement, suggesting that these compounds cross-reacted in the RIA with the same avidity as the Δ^9-THC used to calibrate the assay. Chromatograms obtained before and after treatment of the rabbit urine with β-glucuronidase and sulphatase were the same, suggesting that these enzymes did not produce hydrolysis.

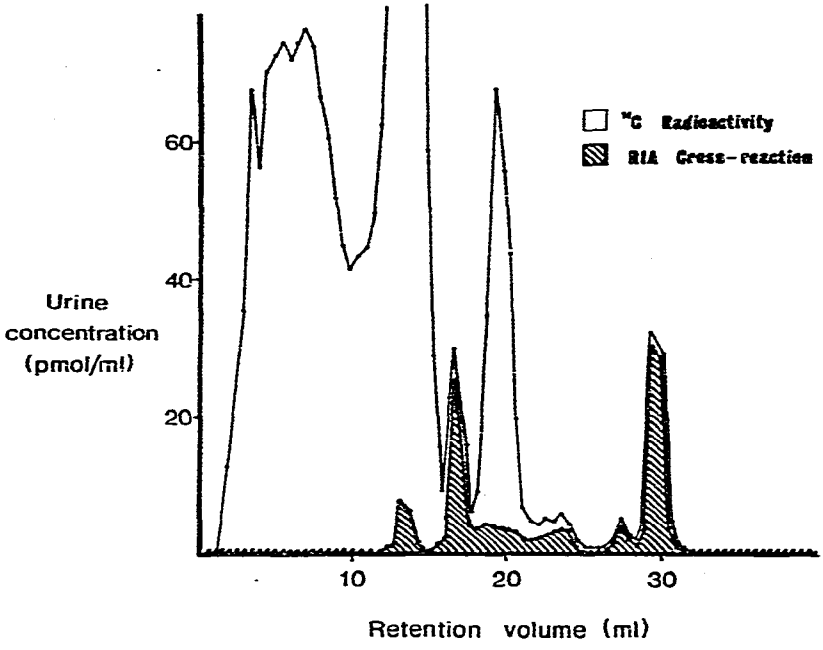

Fig. 2. HPLC elution patterns of radiolabelled Δ^9-THC metabolites and cross-reacting Δ^9-THC metabolites present in a hydrolysed rabbit urine sample.

Chromatograms of the cross-reacting metabolites from the human urine samples obtained after smoking Δ^9-THC impregnated cigarettes were similar to those obtained from the rabbit urine. Unhydrolysed human urine samples contained the cross-reacting components at retention volumes 13.5 and 16.5 ml (Fig. 3a). The latter component had a retention volume equivalent to Δ^9-THC-11-oic acid and in some samples made a major contribution to the level of cross-reaction. Hydrolysed urine gave the three components at 13.5, 16.5 and 30 ml (Fig. 3b) again observed with the rabbit urine sample.

The HPLC–RIA procedure was used to isolate, for mass-spectral identi-

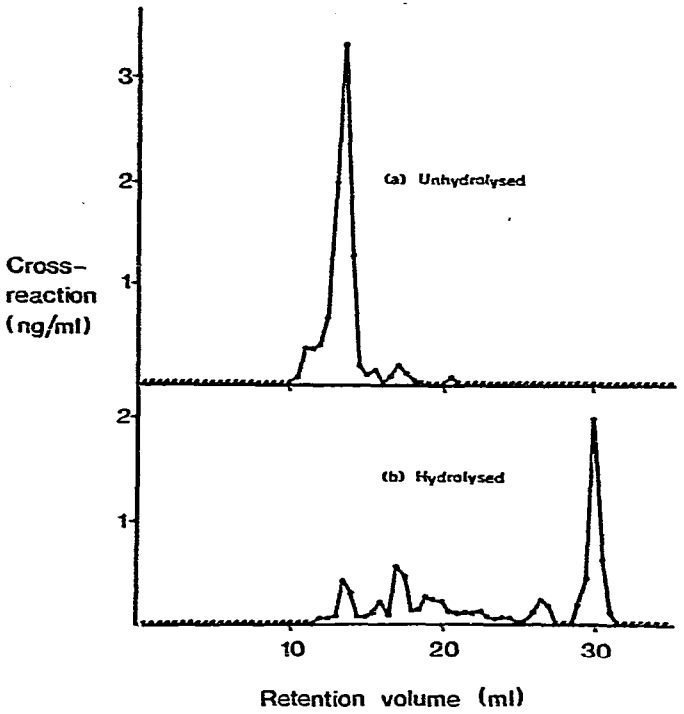

Fig. 3. HPLC–RIA chromatograms of an (a) unhydrolysed and (b) hydrolysed urine sample from subject 3 for the period between 1 and 2 h after smoking Δ^9-THC (8 mg).

fication, the two components eluting at retention volumes 16.5 and 30 ml from a hydrolysed sample of human urine. The sample used was one submitted for forensic analysis and which contained a high level of cross-reacting material (680 ng/ml) and gave an HPLC–RIA chromatogram (Fig. 4) similar to those obtained from other samples in which cannabinoid metabolites were present.

The mass spectrum of the TMS derivative of the component with HPLC retention volume 16.5 ml (GC retention time = 2.5 min) gave major ion peaks at *m/e* 488, 473 (loss of $-CH_3$) and 371 (loss of –COOTMS) and the methylated derivative (GC retention time = 5 min) at *m/e* 358, 343 (loss of $-CH_3$) and 299 (loss of $-COOCH_3$). The chromatographic retention indices and mass spectra were found to be identical to those of the TMS and methyl ester derivatives of Δ^9-THC-11-oic acid.

The mass spectrum of the component with HPLC retention volume 30 ml (GC retention time = 5 min) gave major ions at *m/e* 358, 343 (loss of $-CH_3$) and 299 (loss of $-COOCH_3$) identical to that of methylated Δ^9-THC-11-oic acid. The mass spectrum of the TMS derivative (GC retention time = 2 min) had major ions at *m/e* 430, 415 (loss of $-CH_3$) and 371 (loss of $-COOCH_3$) and was also identical to the TMS derivative of the methyl ester of Δ^9-THC-11-oic acid. The cross-reacting component with a retention volume of 16.5 ml was therefore Δ^9-THC-11-oic acid and the component with retention volume 30 ml, its methyl ester.

Hydrolysis of the urine with ethanolic sodium hydroxide (1 *M*) in place of the methanolic sodium hydroxide solution normally used for conjugate hydrolysis again

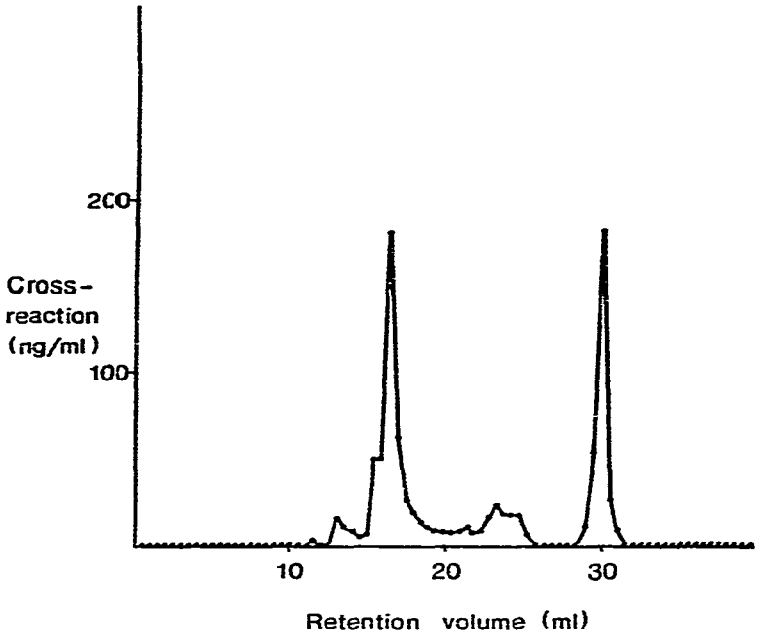

Fig. 4. HPLC–RIA chromatogram of the hydrolysed urine sample used as the source of metabolites for mass-spectral identification.

resulted in the formation of a cross-reacting component with HPLC retention volume 30 ml. The mass spectrum of this compound was that of the ethyl ester of Δ^9-THC-11-oic acid with major ions of 372, 357 (loss of $-CH_3$) and 299 (loss of $-COOC_2H_5$). Δ^9-THC-11-oic acid itself is not esterified in alcoholic sodium hydroxide at room temperature and the metabolite ester was therefore probably formed by trans-esterification from a urinary Δ^9-THC-11-oic acid conjugate. Δ^9-THC-11-oic acid methyl ester slowly hydrolyses in methanolic sodium hydroxide so that the amount of Δ^9-THC-11-oic acid observed after alkaline hydrolysis of a urine sample would be a product of this hydrolysis and that originally present as unconjugated metabolite.

The major cross-reacting cannabinoid components in urine with the antiserum used in this study appear, therefore, to be an unidentified conjugate of Δ^9-THC-11-oic acid with some unconjugated metabolite. The conjugate is responsible for the major area of cross-reaction observed in HPLC–RIA chromatograms of unhydrolysed urine and most of the conjugate is converted with methanolic sodium hydroxide to Δ^9-THC-11-oic acid and its methyl ester. Smaller concentrations of other unidentified cross-reacting components have also been observed in hydrolysed urine as well as major metabolites in rabbit urine which did not cross-react in the assay.

A consistent HPLC elution pattern of urinary cross-reacting components has been observed for samples containing Δ^9-THC metabolites. The HPLC–RIA measurement of the three major cross-reacting compounds in alkaline hydrolysed samples provides a sensitive and specific method for cannabinoid metabolite detection and is suitable, with RIA itself as a screening procedure, for the routine analysis of samples submitted for forensic examination. The technique is potentially suitable for the

detection of further cannabinoid metabolites by employing an antiserum of wider specificity.

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