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DIRECT INJECTION OF URINE ON A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC COLUMN-SWITCHING SYSTEM FOR DETERMINATION OF Δ^9-TETRAHYDROCANNABINOL-11-OIC ACID WITH BOTH ULTRAVIOLET AND ELECTROCHEMICAL DETECTION

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SUMMARY

A method is presented for determination of Δ^9-tetrahydrocannabinol-11-oic acid in urine by means of a fully automated liquid chromatographic system. Aliquots (200 μl) of hydrolysed urine from prison inmates were directly injected onto a pre-column, followed by chromatography on two columns with different selectivity: CN and C_8 columns. To obtain both greater selectivity and a low detection limit a twin-detector principle was used, consisting of both ultraviolet and electrochemcial detection. Urine samples found to be positive with the EMIT cannabinoid were analysed, and the results were compared with those obtained from a well established gas chromatographic–mass spectrometric method. The precision of the method was 2.8% at a mean concentration of 85 ng/ml and 13.4% for 6 ng/ml of the acid. The detection limit was below 5 ng/ml.

INTRODUCTION

Abuse of cannabis is mainly established by identification of its acid metabolite, Δ^9-tetrahydrocannabinol-11-oic acid (THC acid), in urine. For screening purposes the EMIT cannabinoid d.a.u. or s.t. assay [1] is widely used. These immunological tests are inherently unspecific, and positive results should, therefore, be confirmed by a more specific method. Furthermore, to indicate when cannabis was last taken in relation to urine sampling, quantitative methods are needed to determine individual metabolites [2].

A few high-performance liquid chromatographic (HPLC) methods based on UV detection [3] or radioimmunoassay (RIA) [4] and gas chromatographic methods with flame-ionization detection [5] or electron-capture detection [6] have been reported, but they either lack sensitivity or specificity, or involve a

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long clean-up procedure before analysis. Another method combining HPLC and GC [7] gave a high degree of selectivity, but was limited by the laborious sample treatment, which made it unsuitable for routine use.

At our laboratory a gas chromatographic–mass spectrometric (GC–MS) method[8, 9] has been used for confirmation, as well as for quantitative determinations of THC acid in urine. However, because of the large number of samples, the GC–MS method was time-consuming owing to a tedious clean-up procedure. Therefore, an HPLC method was worked out, which eliminated the need for derivatizaton and further simplified the clean-up procedure by offering the use of direct injection of urine onto the chromatographic system. Applications of a similar HPLC system have been described elsewhere [10, 11].

This paper describes an HPLC method for determination of THC acid in the urine from prison inmates. The method involves a column-switching technique to obtain chromatographic separations on two different columns in series, and with both UV detection and electrochemical detection (ED).

EXPERIMENTAL

Chemicals

THC acid was purchased from Alltech Applied Science Labs. (Deerfield, IL, U.S.A.). The eluents were prepared from orthophosphoric acid (85%, Suprapur; Merck, Darmstadt, F.R.G.) and acetonitrile (HPLC grade; Fisons, Loughborough, U.K.). Deionized water was purified by filtration through an Elgastat UHQ (ELGA, Wycombe, U.K.). Acetic acid (100%), methanol and potassium hydroxide were of analytical grade and obtained from Merck.

Equipment

The main parts of the HPLC system consisted of one M590 and two M45 pumps (Waters Assoc., Milford, MA, U.S.A.), a Waters automated valve station (WAVS), an M481 UV detector (Waters) and a Model 5100A Coulochem electrochemical detector (ESA, Bedford, MA, U.S.A.) equipped with a Model 5010 analytical cell and a Model 5020 guard cell. An ISS-100 autosampler (Perkin-Elmer, Norwalk, CT, U.S.A.) and a C-R3A integrator (Shimadzu, Kyoto, Japan) were incorporated in the system.

Analytical conditions

Pre-column 1: Guard-Pak™ cartridge, CN column (Waters); eluent A: 40 m*M* phosphoric acid; column 2: 70×4.0 mm I.D., Nucleosil CN, 5 μm (Macherey-Nagel, Düren, F.R.G.); eluent B: acetonitrile–40 m*M* phosphoric acid (1:2, v/v); column 3: 150×4.0 mm I.D., Nucleosil C_8, 5 μm (Macherey-Nagel); eluent C: acetonitrile–40 m*M* phosphoric acid (1:1, v/v). Flow-rates were 1.0 ml/min throughout.

The electrochemical detector was set at 0.30 V (precondition) and 0.60 V (quantitation potential); the gain was set at 40×10 and the guard cell at 0.90 V. The UV detector was set at 220 nm.

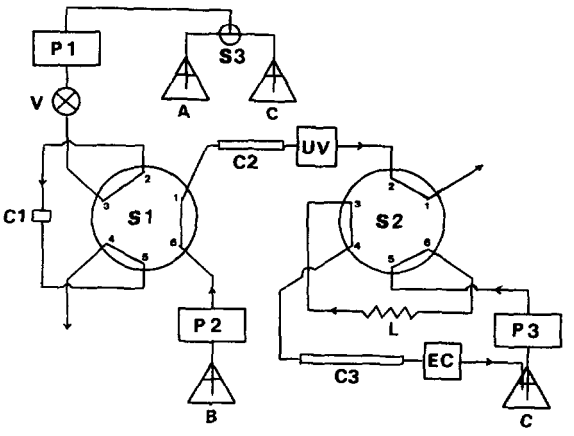

Fig. 1. HPLC system. P1, P2 = M45 pump; P3 = M590 pump; UV = UV detector; EC = electrochemical detector; V = autosampler; L = 1-ml loop; C1 = Guard-PAK CN column; C2 = CN column; C3 = C_8 column; S1, S2 = high-pressure valve; S3 = low-pressure valve. All valves in the WAVS unit.

Sample preparation

A sample of urine (1 ml) was heated with saturated potassium hydroxide in methanol (100 μl) in a screw-cap glass tube for 15 min at 110°C. After the pH of the hydrolysed urine had been adjusted to 4–5 with 0.3 ml of concentrated acetic acid, the sample was transferred to an injection vial.

Automated clean-up and analysis

The whole HPLC system is outlined in Fig. 1. All further steps, including the clean-up and the analysis, were fully automated. The M590 pump (P3) was used to control the valves in the WAVS unit, and the time events program is listed in Table I. Apart from the M590 pump, two M45 pumps were used, one pump (P1) for eluents A and C, and the other pump (P2) for eluent B. An external pulse

TABLE I

TIME EVENTS PROGRAM FOR CONTROLLING THE WAVS THROUGH THE M590 PUMP

Events 1, 2 and 3 refer to the valves S1, S2 and S3, respectively; F = OFF (see Fig. 1, valve S1); N = ON (valve S2).

Segment No.	Time (min)	Event							
		1	2	3	4	5	6	7	8
1	0.00	F	N	F	F	F	F	F	F
2	2.00	N	N	F	F	F	F	F	F
3	3.00	F	N	N	F	F	F	F	F
4	6.00	F	N	F	F	F	F	F	F
5	8.10	F	F	F	F	F	F	F	F
6	8.80	F	N	F	F	F	F	F	F

TABLE II

BASIC AND TIME PROGRAMS FOR CONTROLLING THE SIGNAL OUTPUT FROM THE TWO DETECTORS

BASIC program		Time program	
10	REM UV IS ATTACHED TO C-R3A	0.01	L. ON
20	A% = 04H: OUT 1, A%	0.02	RUN 10
30	ATTEN(FILE) = 5	0.03	PRINT DATE$, TIME$
40	SPEED (FILE) = 5	7	L. OFF
50	SLOPE(FILE) = 1000	14.9	L. ON
60	GOTO 200	15	RUN 100
100	REM EC IS ATTACHED TO C-R3A	15.2	L. OFF
110	B% = 010H: OUT 1, B%	29.5	ZERO
120	ATTEN(FILE) = 2		
130	SPEED(FILE) = 2.5		
140	SLOPE(FILE) = 1000		
200	END		

damper and the guard cell (omitted in Fig. 1) were installed in the line between the M590 pump and the switching valve (S2). The basic steps involved in the on-line clean-up procedure were:

(1) An aliquot (200 μl) of the sample was injected onto the pre-column (C1), where the THC acid was enriched, and at the same time fast-eluting urinary compounds were washed out. The eluent used was A.

(2) After 2 min, the position of the valve (S1) was altered and the THC acid eluted onto the CN column (C2) with eluent B.

(3) After 3 min (from injection), the elution was finished when the valve S1 was activated. Simultaneously, the valve S3 was altered and the pre-column washed with eluent C for 3 min, before re-equilibration with eluent A. During this time the THC acid was chromatographed on the CN column.

(4) Before the effluent containing the THC acid reached the UV detector, the valve S2 was altered so that the eluate went through the loop L. Once all the THC acid had passed into the loop the valve S2 was reactivated so that the contents of the loop were pumped through column C3 by means of pump P3.

The total analysis time (30 min) was divided into two periods of 15 min. During the first period, the integrator input was connected to the UV detector, then for the remaining 15 min to the electrochemical detector. This was carried out by incorporating a digital I/O option board to the C-R3A integrator. Through the use of this board, either of the signal outputs from the two detectors can be connected to the integrator, one at a time, by means of relays. These relays in turn were controlled by a simple basic program, which was activated in the time program. Both programs are listed in Table II.

Gas chromatography–mass spectrometry

An earlier GC–MS method [8] was modified [9] as follows. The GC–MS system (a gas chromatograph HP 5890, a mass detector HP 5970 and a data system

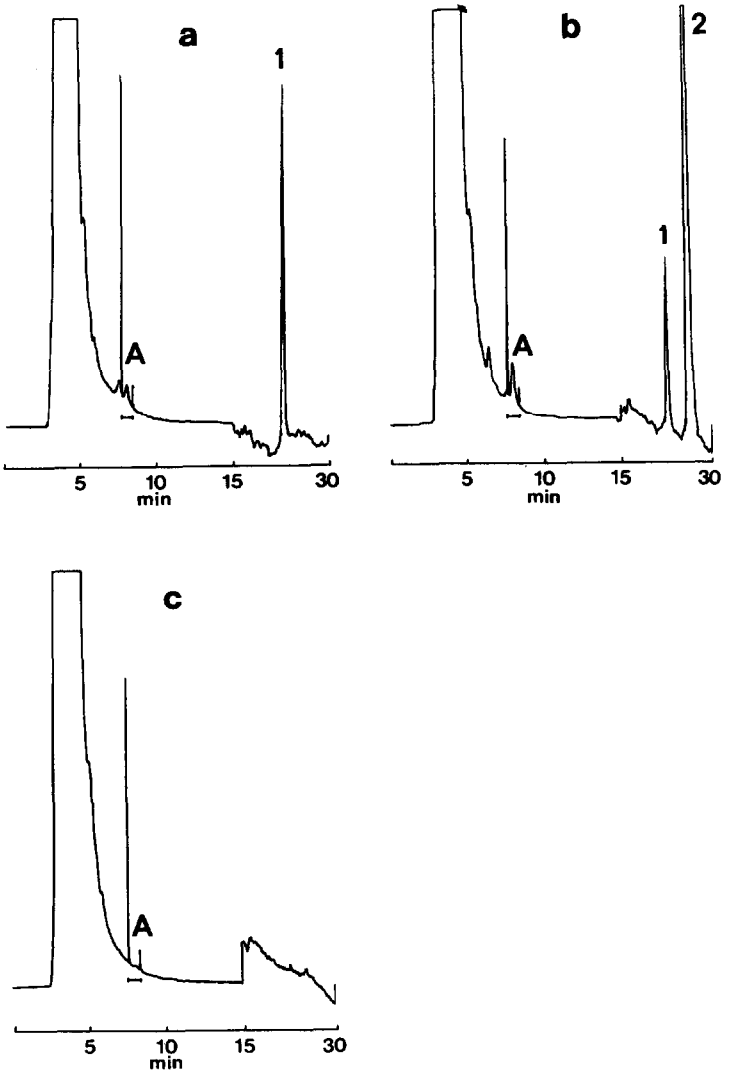

Fig. 2. Chromatograms from the analysis of authentic urine samples. The measured concentrations of THC acid were (a) 32 ng/ml, (b) 14 ng/ml and (c) "negative". A = zone trapped. Peaks: 1 = THC acid; 2 = unknown constituent. Detection mode: UV for retention times 0–15 min; electrochemical for retention times 15–30 min.

HP 9816, all from Hewlett-Packard, Palo Alto, CA, U.S.A.) was operated in the electron impact mode, and the ions monitored were: *m/e* 622 and *m/e* 459 from the pentafluoropropyl pentafluoropropionyl derivative of THC acid and *m/e* 625 and *m/e* 462 from the deuterated analogue (internal standard). Splitless injections were made on a 15 m×0.25 mm I.D. fused-silica DB-5 column (J&W Scientific, Rancho Cordova, CA, U.S.A.). The injector temperature was 250°C. The oven temperature was programmed as follows: 150°C for 2 min, then raised by

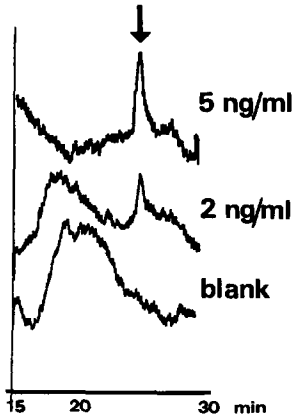

Fig. 3. Analysis of urine samples spiked with 2 and 5 ng/ml of THC acid together with a blank urine sample showing the limit of detection. Detection by the electrochemical detector. Arrow indicates the retention time of THC acid.

45°C/min to 280°C. For quantitative measurements a calibration curve, based on the area ratio of *m/e* 622 and *m/e* 625, was established.

RESULTS

Chromatography

Fig. 2 shows chromatograms from the analysis of urine samples subjected to the clean-up procedure. The concentrations of THC acid (Fig. 2a and b) were 32 and 14 ng/ml, respectively. Both the UV (during the first 15 min) and the electrochemical response (the next 15 min) are plotted in the same chromatogram. Fig. 2b illustrates the different selectivities of the two columns. Although one peak was trapped (zone A), two peaks appeared in the latter part of the chromatogram, one representing THC acid (1) and the other an unknown constituent (2).

Linearity, precision and detection limit

The linearity of the method was established by the analysis of urine samples spiked with known amounts of THC acid ranging from 20 to 200 ng/ml. Each concentration level consisted of replicate determinations ($n=3$) made on different days to establish the reproducibility of the method. The calibration curve, based on the electrochemical response and peak-area measurements, had a correlation coefficient of 0.998. Because the concentration of THC acid found in actual urine specimens can range from a few nanograms to several micrograms per millilitre, a second curve was established. This higher curve, based on the UV detection, was linear from 200 ng/ml to 2 μg/ml with a correlation coefficient of 0.998.

The precision of the method was determined by the analysis of two pools of

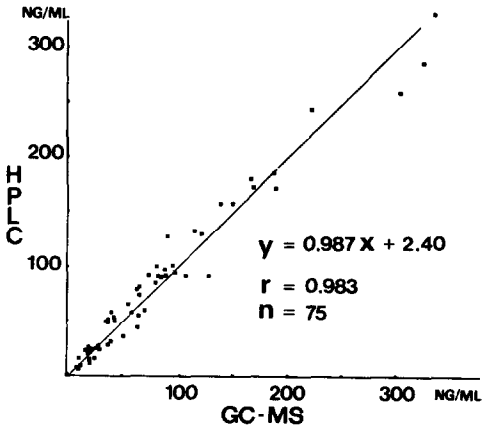

Fig. 4. Scatter diagram showing the correlation between the results of analyses of urine samples by HPLC and GC–MS.

authentic urine with measured THC acid concentrations of 6 and 85 ng/ml. The relative standard deviations (R.S.D.) for the within-day assay were 13.4% for the 6 ng/ml and 2.8% for the 85 ng/ml solution ($n=5$). The R.S.D. for the day-to-day assay was 8.5% for the 85 ng/ml level ($n=5$).

The limit of detection was 2 ng/ml at a signal-to-noise ratio of 3:1, (Fig. 3) which corresponds to ca. 280 pg of THC acid injected.

Correlation with the GC–MS method

Urine samples, determined as positive by the EMIT cannabinoid d.a.u. test, were analysed by the present HPLC method and the modified GC–MS method [8] (see Experimental). The results (Fig. 4) showed good agreement, and the correlation coefficient was 0.983 ($n=75$). Out of 75 urine samples, four were found to be negative by both the GC–MS and the HPLC method.

DISCUSSION

Comparison with other methods

Of the methods reported, none has such a simple sample treatment before analysis as the present HPLC method. Moreover, the HPLC method combines a high degree of selectivity with a low detection limit, almost comparable with most GC–MS methods. Among non-MS methods, only a few have reported a detection limit below 5 ng/ml, one is the HPLC–RIA method [4] and the other a GC method with electron-capture detection [6]. The HPLC–RIA method, however, requires a long analysis time leading to a throughput of only six samples each day. A major disadvantage with all GC methods [5–7] is the need for several time-consuming extraction and derivatization steps before chromatography.

Electrochemical detection

THC acid is a phenolic compound and therefore, as reported [12], undergoes oxidation at the electrode surface. Unfortunately, urine contains many endogenous substances that are also oxidizable. However, by working with the column-switching technique, only the relevant part of the first chromatogram is injected onto the second column. This limits the amount of polar oxidizable substances that would otherwise cause contamination of the analytical cell resulting in a lower detector response.

During the initial start-up of the electrochemical detector it is essential to equilibrate the system by recycling the eluent C for at least two to three days before injecting any biological samples. The background current is accordingly stabilized to a level less than 1 μA. Because both the guard cell and the analytical cell have a self-cleaning effect on the eluent, the possibility exists to recycle the eluent even during analysis of samples. After a couple of weeks of recycling, the background current is normally ca. 0.5 μA. Because of the recycling procedure, the composition of the eluent C after prolonged use will gradually change, owing to injection of the sample volume trapped into the sampling loop (L). Accordingly, the retention time of THC acid on the C_8 column will be altered. However, this is a delayed effect and will not influence the result within a series of samples or between neighbouring series. Another drawback is that injection of a sample with a solvent composition different from that of the eluent often leads to a noisy baseline, especially when a sensitive electrochemical detector is used. This does occur, but not in the part of the chromatogram where THC acid appears. A useful modification is to connect the two waste lines to the two remaining solvent valves in the WAVS unit. Of the two lines out from each valve, one is led back to the solvent reservoir and the other to a waste bottle. By this configuration the eluents A and B can be recycled after the last sample in a series. This serves to reduce the solvent consumption and the work involved in preparation of eluents.

Chromatography

The pre-column serves the purpose of trace enrichment of THC acid. This column is easily changeable. The shorter analytical CN column was found to be ideal for separating fast-eluting urinary compounds from THC acid, whereas the longer analytical C_8 column, with a greater separating capacity, was useful for the final chromatography before ED.

Even after more than 100 injections of urine there is no decrease in performance of the HPLC system. It must be stressed, however, that an accurate and precise determination of the "window" for trapping the eluate into the sampling loop (1 ml) is essential for the overall result. If the window is kept too small and a minor change in retention time occurs, the THC acid may not be injected onto the C_8 column. On the other hand, a large window results in a larger injected volume, which leads to peak broadening and a loss of efficiency. The selectivity is also reduced. Before a series of urine samples is analysed the retention time is

controlled by injection of a standard solution of THC acid and, if necessary, the window is adjusted accordingly. For such a window determination, the UV detector incorporated between the first analytical CN column (C2) and the valve (S2) is essential. However, when a very high concentration of THC acid is present in a urine sample the electrochemical detector is overloaded, leading to an incorrect quantitation. In this case two possibilities exist: (a) quantitate the THC acid by means of the UV detector; (b) re-analyse and either inject a smaller volume of urine or reduce the gain on the electrochemical detector. If option (a) is used, it must be deduced that the peak in zone A is only THC acid. This is easily done by evaluation of the second part of the chromatogram – and if there are two peaks, as in Fig. 2b, the only way to quantitate the THC acid is alternative (b).

The reproducibility of the method is good enough to warrant the use of external standards. For routine work a calibration urine sample at 100 ng/ml should be analysed to check the overall performance of the method.

A disadvantage of the HPLC method is that the sample throughput is relatively low (two urine samples per hour) owing to the long time needed for chromatography. This means that an autosampler working 24 h a day is a mandatory requirement for handling a large batch of samples.

In conclusion, the HPLC method offers the same limit of detection as a GC–MS method previously reported and gives excellent selectivity, with the minimum of sample pre-treatment.

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