ELSEVIER

Journal of Chromatography B, 677 (1996) 245–255

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Determination of Δ^9-tetrahydrocannabinol from human saliva by tandem immunoaffinity chromatography–high-performance liquid chromatography

V. Kircher[a], H. Parlar[b,*]

[a]*Department of Analytical Chemistry, University of Kassel, H.-Plett-Strasse 40, 34132 Kassel, Germany*
[b]*Department of Chemical Technical Analysis, Technical University of Munich, 85350 Freising-Weihenstephan, Germany*

Received 6 April 1995; revised 27 September 1995; accepted 10 October 1995

Abstract

Smoking or ingestion of cannabis causes cognitive, perceptual and behavioural changes, which are responsible for impaired performance in driving motor vehicles. In this paper a novel liquid chromatographic assay for the selective quantification of Δ^9-tetrahydrocannabinol, the major indicator of a present cannabis intoxication in saliva, is described. The method involves a column-switching procedure and requires an extremely simple pre-treatment of the sample. Deproteinized saliva was directly injected into the chromatographic system. The clean-up and enrichment procedure was performed in an immunoaffinity column, followed by the transfer of the antigens to an octylsilica analytical column. The immunoaffinity sorbent was obtained by covalent immobilization of specific antibodies on epoxy-activated silica. The mobile phase consisted of methanol–aqueous 0.15 mol/l NaCl solution (elution programmed) and the analyte was detected by measuring the UV absorption at 220 nm. Using an injection volume of 4.5 ml (dilution 3:2, v/v) the limit of quantification was 20 ng/ml, at a signal-to-noise ratio of 5. Recoveries were estimated to be in the range of 70%. Both intra- and inter-day coefficients of variation were below 5%

Keywords: Δ^9-Tetrahydrocannabinol

1. Introduction

As a result of the Single Conventions Treaty (1961), most members of WHO enacted national controlled substance acts in the 1960s and 1970s. Besides other narcotic drugs, cannabis and its major psychoactive component Δ^9-tetrahydrocannabinol (Δ^9-THC; see Fig. 2A) were appointed to class I to prohibit their general use. Nevertheless, numerous studies concerning the pharmacokinetic and pharmacologic properties of cannabis have reported some beneficial therapeutic effects (e.g. antiemetic, anticonvulsive and analgesic) of several cannabis components (cannabinoids), as well as a low tendency to produce physical and psychical dependence, compared with other psychotropic drugs [1–3]. Over the last decade, some nations have amended their legislation towards cannabis consumption and medical usage. Meanwhile, Δ^9-THC was appointed to class II in the USA (1984) and Germany (1992). Moreover, cultivation, acquisition and possession of the drug in small quantities and for own use was

*Corresponding author.

0378-4347/96/$15.00
SSDI 0378-4347(95)00451-3

permitted in Spain (1979) and the Netherlands (1982) or characterized by limited impunity in Germany (1992). This new jurisprudence has affected established analytical strategies in forensic chemistry. While up to this time cannabis abuse had to be identified by detection of long-term appearing cannabinoid metabolites in urine (positive results from immunoassay screening tests had to be confirmed by chromatographic analysis of 11-COOH-Δ^9-THC, the major THC metabolite), today's prosecution is focused on large-scale drug trade and dope pedlars in these countries.

On the other hand, drugged driving is prohibited by traffic regulations in most nations and has to be attested by the analysis of narcotic compounds in body fluids. However, the quantification of Δ^9-THC in blood is very difficult, because of its low concentrations, and the screening of THC metabolites in urine, sweat or hair cannot reflect the degree of a present cannabis intoxication because of the slow appearance of the compounds in these compartments. On this basis, saliva is the most suitable matrix to answer this forensic question. After the administration of typically 5–20 mg Δ^9-THC, the level of active substance in saliva reaches more than 1000 ng/ml, and is coincident with maximum psychotropic effects. When the intoxication has faded after 3–4 h, the concentration has diminished to about 50 ng/ml [4–6]. The measurement of cannabinoids in saliva presents several other advantages over measurements in blood (simple and easy sample collection, a non-invasive and stress-free experience for the subject).

Most of the previously reported methods for cannabinoid analysis in body fluids suffer from complex and time-consuming extraction procedures (LLE or SPE), posing problems of selectivity during the following chromatographic and detection steps [7–28]. Immunoaffinity chromatography (IAC) presents higher efficiencies for selective enrichment and has since long been used for preparative separation of biogenetic macromolecules [29–33]. Far less applications have been published for antigens smaller than 5000 (M_r), the so-called haptens, such as 17-β-estradiol [34], aflatoxins [35] or ecdysteroids [36]. Because of difficulties in a fine control of sorption and desorption phenomena IAC is often unable to resolve structurally related components recognized in the sample. Usually, all these cross-reactants are eluted in one fraction, as illustrated in Fig. 1. In cases of acute intoxication with cannabis more than 60 cannabinoids with analogous structures, as well as their metabolites, are supposed to be in saliva. While the majority can show high immunologic cross-reactivity only few of them produce psychotropic effects, so that the results of immunological methods in general cannot reflect the degree of intoxication.

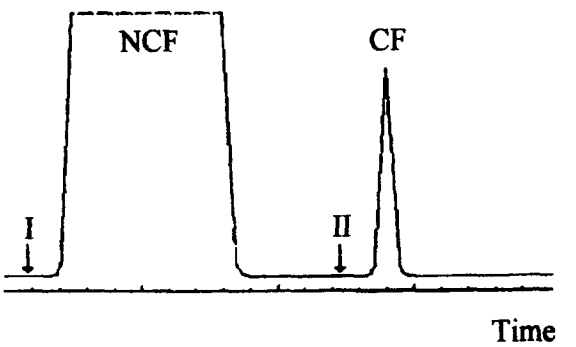

Fig. 1. Typical immunoaffinity chromatogram. Step I: injection; step II: sharp enhancement of eluotropy (desorption); NCF, non-cross-reacting fraction of sample components; CF, cross-reacting components

The strategy of the HPLC method described in the present paper was to use IAC merely for selective extraction of cannabinoids from saliva samples and to resolve the resulting fraction of desorbed compounds with an on-line coupled RP column for specific Δ^9-THC quantification. The IAC stationary phase was obtained by covalent immobilization of cannabinoid-specific IgG antibodies on epoxy-activated silica. For published concentrations of Δ^9-THC in human saliva during cannabis psychotropic effects, blank samples were spiked with THC and the other major (but non-psychotropic) cannabinoids, cannabinol (CBN) and cannabidiol (CBD) (Fig. 2), in the concentration range between 10 ng/ml and 1000 ng/ml for recovery experiments.

2. Experimental

2.1. Chemicals and reagents

Δ^9-THC, CBN and CBD were purchased from Sigma (Deisenhofen, Germany). Supelcosil Epoxy

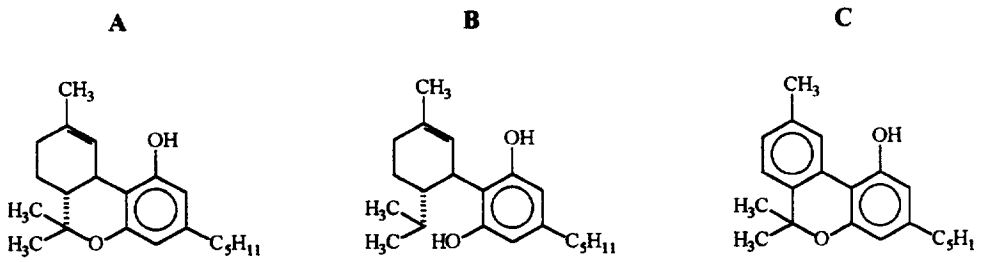

Fig. 2. Chemical structures of Δ^9-tetrahydrocannabinol (A), cannabidiol (B) and cannabinol (C). IUPAC nomenclature for Δ^9-THC: (6a *R-trans*)-6a,7,8,10a-tetrahydro-6,6,9-trimethyl-3-pentyl-6*H*-dibenzo[*b*,*d*]pyran-1-ol; CBD: (1 *R-trans*)-2-[3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol; CBN: 6,6,9-trimethyl-3-pentyl-6*H*-dibenzo[*b*,*d*]pyran-1-ol.

540 (particle size, 40–90 μm; average pore diameter, 50 nm; epoxy groups, 300 μmol per g of dry gel) was provided by Supelco (Bad Homburg, Germany). THC-specific antiserum (sheep; Lot No. J 912; specific IgG titre, 14 mg per ml of serum) was purchased from Guildhay (London, UK). The antiserum was specified with the following cross-reactivities: Δ^9-THC, 100%; CBN, 6.3% and CBD, 0.3%. Distilled water was purified by passage through a Milli-Q II treatment system (Millipore, Bedford, MA, USA). Methanol of HPLC grade was purchased from Baker (Deventer, Netherlands). Other reagents were of analytical grade and were obtained from the following sources: 2-aminoethanol and denatured ethyl alcohol (Aldrich, Steinheim, Germany); acetic acid (glacial), perchloric acid (70%), potassium phosphate (monobasic), sodium acetate, sodium chloride, sodium phosphate (dibasic) and sodium sulphate (Fluka, Neu Ulm, Germany).

2.2. Preparation and packing of the affinity gel

Supelcosil Epoxy (1.6 g) was added to a solution of 100 μl THC antiserum in 10 ml coupling buffer (1.5 *M* Na_2SO_4 in 0.1 mol/l phosphate buffer; pH 7) and the mixture was stirred gently at room temperature. At time intervals of 30 min the absorption of the upper cleared up phase was measured at 280 nm (spectrophotometer Philips PU 8700; Pye Unicam, Cambridge, UK) to determine the degree of immobilization. After 240 min the reaction was stopped. The conjugate was sucked now through a fritted glass disk funnel and rinsed three times with 10 ml of cleansing solution A (0.1 mol/l acetate buffer; pH 4) and B (0.05 *M* NaCl in 0.1 mol/l phosphate buffer; pH 7) in turn. Subsequently, the gel was dispersed in 10 ml solution of 0.2 *M* 2-aminoethanol in coupling buffer and stored for 3 days at 4°C to block the remaining epoxy groups. Afterwards, the gel was sucked and rinsed in the same manner as described above. Suspended in cleansing buffer B the affinity matrix was slurry-packed into a stainless-steel HPLC cartridge (125 × 4 mm I.D.; Merck, Darmstadt, Germany). Last, the column was purged with 250 ml of 0.15 mol/l NaCl solution for equilibration and was stored at 4°C until further use. It is important to note that the gel was kept in a wet condition permanently because desiccation can alter the immunological activity.

2.3. HPLC equipment and chromatographic conditions

The HPLC system consisted of two dual-piston pumps, a Model 510 and a Model 501 from Waters Assoc. (Milford, MA, USA), a Model 7125 syringe loading sample injector (Rheodyne, Cotati, CA, USA) equipped with a 5-ml sample loop, a switching valve (Model 7000, Rheodyne) and a Model Lambda Max 481 UV–Vis detector (Waters Assoc.) set at 220 nm. Data acquisition, processing and pump control were performed using a Baseline 810 V 3.02 chromatography data system (Waters Assoc.) operating on an IBM XT computer. The IAC column described above was used for enrichment of cannabinoids. Separation was carried out on a RP-8 column (LiChrospher select B, 125 × 4 mm I.D., 5 μm average particle size; Merck). The mobile phase solvents were (A) methanol–0.15 mol/l aqueous NaCl solution (20:80, v/v) and (B) methanol–0.15

Table 1
Gradient program and sequence of time events

Step	Time (min)	Eluent A (%)	Eluent B (%)	Position switching valve
I	0–5.0	100	0	A
II	5.1–25.0	0	100	B (after 14 min)
III	25.1–35.0	100	0	A (after 34 min)

Eluents were (A) methanol–0.15 mol/l aqueous NaCl solution (20:80, v/v) and (B) methanol–0.15 mol/l aqueous NaCl solution (82:18, v/v). Gradient was performed using a flow-rate of 0.8 ml/min.

mol/l aqueous NaCl solution (82:18, v/v). The elution program (shown in Table 1) was set such that eluent A was maintained for 5 min and 100% of eluent B was reached in 0.1 min at a flow-rate of 0.8 ml/min and at ambient temperature. Injections of saliva and aqueous samples were performed using 5-ml high-performance syringes (Hamilton, Reno, NV, USA). For the injection of calibration standards a HPLC syringe (25 μl capacity; Hamilton) was used.

2.4. Column-switching system

The system and the chromatographic conditions used for on-line sample extraction are shown in Fig. 3 and Table 1. Samples are injected and transported to the IAC column by eluent A in gradient step I (switching valve in position A). Cross-reacting cannabinoids form antigen–antibody complexes, while the other components are eluted in the waste. In gradient step II the complexes are dissociated and the cannabinoid fraction is eluted to the analytical RP column, which is linked to the chromatographic flow

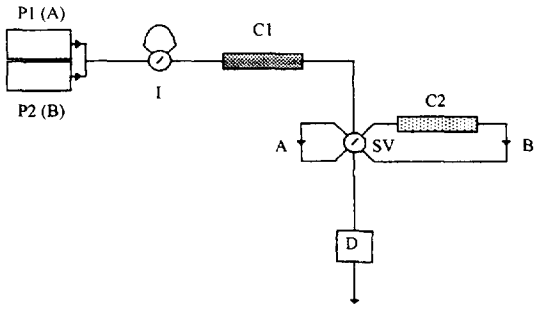

Fig. 3. Schematic diagram of the column-switching system. P1, P2: HPLC pumps; (A), (B): eluents; I: injector; C1: immunoaffinity column; C2: analytical column; SV: switching valve; A, B: positions; D: UV detector.

after 14 min (switching valve in position B). The delay of 9 min between changing of eluents and switching of the valve to position B is caused by the volume of the sample loop and the column C1. Gradient step III is used for pre-conditioning of the IAC column and has no influence on the chromatographic separation of a present run.

2.5. Standard solutions and calibration curves

Stock standard solutions of Δ^9-THC, CBD and CBN were prepared by dissolving the appropriate amounts in ethyl alcohol to give 1000 μg/ml. Analysis of the cannabinoid solutions indicated that the purity was greater than 95% by capillary GC (flame ionization detection) and 100% by reversed-phase HPLC (UV detection). Calibration standards were prepared by diluting the appropriate volume of stock standard to give final concentrations of 0, 1, 2, 3, 5, 10, 25, 50, 100 and 300 μg/ml. They were stored in glass flasks at 4°C. The calibration curves were prepared using the peak area, and the mean value obtained by averaging three measurements of each standard solution (direct injection of 10 μl) was plotted.

2.6. Pretreatment of spiked saliva

Blank human saliva was collected from five male and five female volunteers (strictly non-users of cannabis) 1 h after the moderate consumption of alcohol, coffee and cigarettes. The pooled material was divided into 10-ml units and spiked with calibration standards to produce final concentrations of 0, 10, 20, 30, 50, 100, 250, 500 and 1000 ng/ml of each cannabinoid. Each sample was allowed to stand for 1 h to secure thorough distribution. When the

samples were not being analysed, they were kept frozen at −20°C.

2.7. Sample preparation

In order to protect the IAC column and increase its lifetime, the saliva samples were deproteinized before their injection into the HPLC system. Considering the lipophilic character of the three analytes (partition coefficient (octanol–water) > 10^5 [37]), each sample was added with methanol to minimize unspecific sorption on the surfaces of vials or syringes.

Perchloric acid (40 μl; 35%) and methanol (1 ml) were added to 3 ml of saliva to precipitate proteins. The mixture was shaken for 5 min on a mechanical shaker, neutralized by the addition of 1 ml phosphate buffer (0.1 mol/l; pH 7) and centrifuged at 2000 *g* for 5 min to remove cellular debris and undissolved contaminants. Subsequently, the complete liquid phase was transferred into a disposable polypropylene syringe (5 ml; B. Braun, Melsungen, Germany) and passed through a disposable filter holder Minisart RC 15 (cellulose membrane, average pore size 0.4 μm; Sartorius, Göttingen, Germany). An aliquot of 4.5 ml was injected into the apparatus.

2.8. Extraction recovery and precision

In addition to recovery experiments with the samples described above, two control groups were prepared to determine the intra-system extraction efficiency and the losses caused by sample preparation. Control group CA was composed of saliva spiked with 50, 100 and 500 ng/ml Δ^9-THC, CBD and CBN. For control group CB blank saliva was deproteinized with the procedure described in the Section 2.7 and spiked with 50, 100 and 500 ng/ml of Δ^9-THC, CBD and CBN afterwards. The intra-system recovery was calculated by comparing the peak areas of the control group CB with those of directly injected cannabinoid standard mixtures. The extra-system percentage of losses was calculated by the difference between the extraction yields of group CB and group CA.

The within-day precision was determined by analysing each control of group CA five times on the same day. The day-to-day precision was established by assaying this CA group on three different days.

2.9. Application experiment

A healthy and drug-free male volunteer (weight 70 kg; age 30 years) was administered a mixture of Δ^9-THC, CBD and CBN in 15 ml ethyl alcohol (total amount: 5 mg of each cannabinoid) by oral intake. Eating and drinking was not allowed during this experiment. Seven millilitres of saliva were collected 1 h before administration and 1 h (subjective intoxication), 3 h (the subjective psychotropic effects had faded) and 4 h after administration into 10-ml glass-stoppered centrifuge tubes. They were stored at −20°C before being analysed by the HPLC procedure. Each sample was injected twice.

3. Results and discussion

3.1. Performance of the HPLC system

Only a few methods are available for the measurement of Δ^9-THC in saliva, and they are based on either capillary gas chromatography with electron capture detection or negative ion mass spectrometric detection (both methods require derivatization steps) or liquid chromatography with electrochemical detection as analytical techniques. Furthermore, they all require a time-consuming and non-selective liquid–liquid extraction or solid-phase extraction step. The use of column switching techniques for sample extraction in order to reduce the handling of body fluids seems attractive and has found some applications in clinical analytical chemistry, but mostly by using non-selective stationary phases.

We considered immunoaffinity chromatography to be the best choice for selective sample enrichment in Δ^9-THC analysis. Generally, soft gels are used for the covalent immobilization of specific antibodies in IAC. For the column-switching system we had better experiences with the silica support described in Section 2. The hydrophilic character and the mechanical and chemical stability of the support were shown to be essential for the achievement of immunological effects. In fact, alternatively tested soft gels were somewhat limited by pressure and flow restric-

tions (e.g. Sepharose beads) or caused hydrophobic interactions, which hindered the selectivity (e.g. Toyopearls). These investigations are not reported in this article. The amount of covalently bound IgG antibodies on Supelcosil Epoxy was estimated to be 0.8 mg per 1.6 g of silica (57% of 1.4 mg total specific IgG) by absorption measurements at 280 nm. Pressure on the column at a flow-rate of 0.8 ml/min (gradient steps I and II) was measured at 10.4 MPa. Over several months, more than 250 saliva samples and 200 standard solutions were injected using the same IAC column without reduction in the chromatographic efficiency.

Because antibodies fail to discriminate structurally similar species, two other major cannabinoids (CBD and CBN) were additionally used for analysis to check the ability of the tandem method for selective Δ^9-THC determination. No internal standardization method was performed because of the difficulty in finding an appropriate standard, fitting the following requirements: (a) non-biogenetic origin; (b) cross-reactant on the IAC column; (c) no interference on the analytical column. In preliminary experiments, the eluotropy in gradient steps I and II was optimized to obtain a separation of cannabinoids from UV-absorbing saliva components and a sufficient difference in retention between the cannabinoids, while minimizing the total retention times. Flow-rates higher than 0.8 ml/min were unfavourable because they were associated with a decrease in recovery. The percentage of methanol in gradient step I (20% v/v) was adopted in the sample preparation step to minimize losses caused by sorptions on the surfaces of vials or syringes. Although electrochemical detection is the most suitable method for sensitive quantification of cannabinoids in HPLC we used UV detection to check the selectivity of the IAC enrichment. For optimum sensitivity, the wavelength was set at 220 nm, which was close to the λ_{max} of the three analytes. Under the HPLC conditions described in this paper, no interference from endogenous substance was obtained.

3.2. Chromatograms

The peaks of the three analytes were well resolved and their symmetry was satisfactory. The retention times were 23.2 min for CBD, 24.0 min for CBN and 25.0 min for Δ^9-THC. It should be pointed out that peak symmetry was independent of the injection volume or the concentration of spiked analytes. A systemic peak at 27.0 min appeared in each chromatogram and was unaffected by the eluotropic force in gradient step II. The step-like baseline in Fig. 4, Fig. 5 and Fig. 9 reflects the absorption characteristics of methanol during gradient elution.

Fig. 4 illustrates the results of the analysis of a 10 μg/ml cannabinoid standard mixture using this on-line IAC–RPC method and UV detection. Typical chromatograms of the blank saliva samples and the spiked with Δ^9-THC, CBD and CBN are shown in Figs. 5–7. A chromatogram of real human saliva following the oral administration of a cannabinoid mixture is shown in Fig. 8. Well-defined chromatograms were obtained without interference from other matrix compounds. The selectivity of the column-switching method and the amount of potential interferents on the analytical column are demonstrated in Fig. 9. During the chromatographic run of this blank saliva sample the analytical column was switched permanently in series to the IAC column.

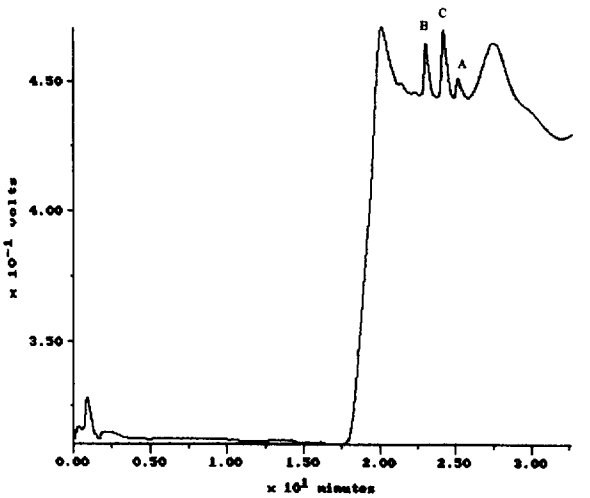

Fig. 4. Chromatogram of a standard mixture of cannabinoids using the on-line IAC-RPC method. Standard mixture containing 10 μg/ml each of Δ^9-THC (A), CBD (B) and CBN (C). For conditions, see Section 2.

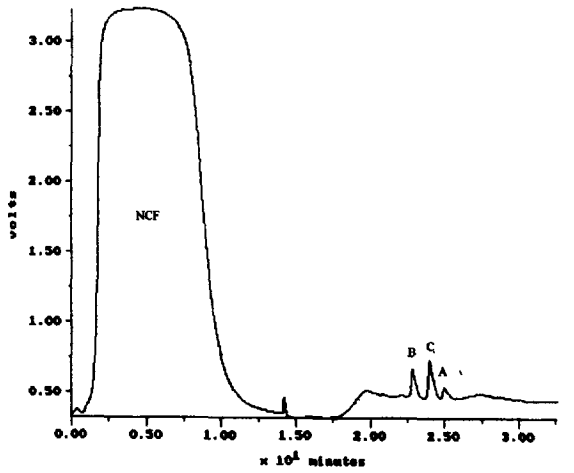

Fig. 5. Chromatogram of a spiked saliva sample containing 500 ng/ml each of Δ^9-THC (A), CBD (B) and CBN (C). NCF, non-cross-reacting fraction. Injection volume: 4.5 ml of deproteinized and diluted (3:2, v/v) saliva. For conditions, see Section 2.

3.3. Calibration, recovery, sensitivity and precision

The minimum detection limits for the determination of standard cannabinoids (injection volume 10 μl) were 2 μg/ml, 1 μg/ml and 0.8 μg/ml for Δ^9-THC, CBD and CBN, respectively, at a signal-to-noise ratio of 3. The calibration curves were linear over the investigated range and gave the following equations: Δ^9-THC, $y = 0.003 + 0.030x$, $r = 0.9998$; CBD, $y = 0.016 + 0.045x$, $r = 0.9998$; CBN, $y = 0.001 + 0.072x$, $r = 0.9999$.

The recoveries of the three cannabinoids on addition of standard solutions to saliva were determined and satisfactory extraction yields were obtained. The results are shown in Table 2. The limits of quantification of saliva were 20, 10 and 10 ng/ml for Δ^9-THC, CBD and CBN, respectively, at a signal-to-noise ratio of 5. Repeated assays of spiked samples (control group CA; see Section 2 indicated that the reproducibility of the procedure was satisfactory over the investigated concentration range. Table 3 summarizes the precision (coefficients of variation) for within-day assay. The day-to-day results are shown in Table 4. The intra-assay C.V. value was less than 5%, as was the inter-assay C.V. value.

For the sample preparation step the choice of a hydrophilic filter membrane (cellulose) was shown to be profitable. Other tested and more hydrophobic membrane materials caused high trapping effects associated with unsatisfactory recoveries below 40%. These included: Anatop 25 (polytetrafluoroethene, pore size 0.1 μm; Alltech Assoc., Deerfield, IL, USA); Millex HV (polyvinylidenefluoride, pore size 0.45 μm; Millipore, Milford, MA, USA) and Minisart NML (cellulose acetate, pore size 0.2 μm; Sartorius).

The intra-system extraction efficiency was determined by assaying control group CB (deproteinized and filtered saliva was spiked; see Section 2). In comparison with control group CA, nearly the same extraction yields were obtained for CBN and Δ^9-THC. Only CBD showed higher recoveries when spiked afterwards. The results are shown in Table 5.

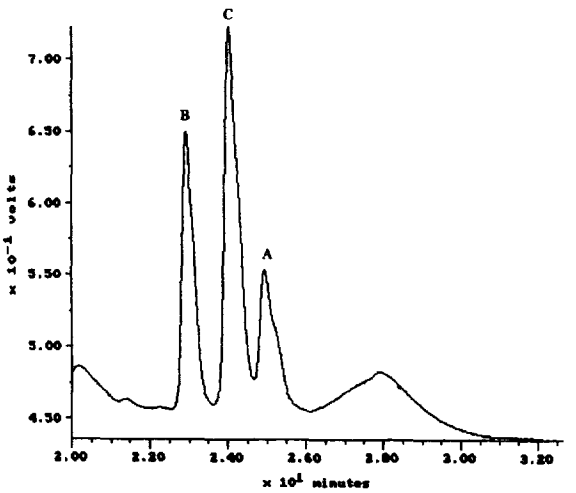

Fig. 6. Chromatogram of a spiked saliva sample containing 500 ng/ml each of Δ^9-THC (A), CBD (B) and CBN (C). Extended segment of Fig. 5.

3.4. Application experiment

This selective assay method was applied to an administration experiment and the three cannabinoids were detectable in saliva up to 3 h after the oral intake of a standard mixture in ethyl alcohol, con-

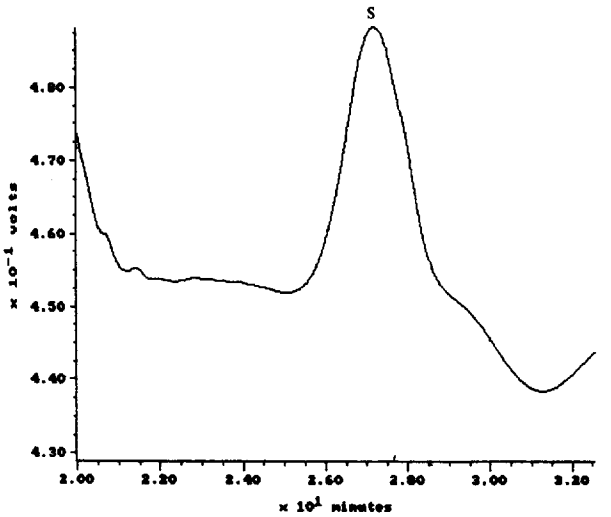

Fig. 7. Chromatogram of a blank saliva sample. Injection volume: 4.5 ml of deproteinized and diluted (3:2, v/v) saliva. Systemic peak (S) at 27.0 min. For conditions, see Section 2

taining 5 mg each of Δ^9-THC, CBD and CBN. The concentrations of the 4 h sample were below the quantification limit. From both positive samples a further chromatographic peak was eluted (component U, see Fig. 8). Considering the selectivity of the IAC column and the comparable short retention time (22.6 min) of the fraction, this phenomenon was attributed to a metabolization of cannabinoids. In fact, additionally to a decline in concentration, a gradual alteration compared with the original elution

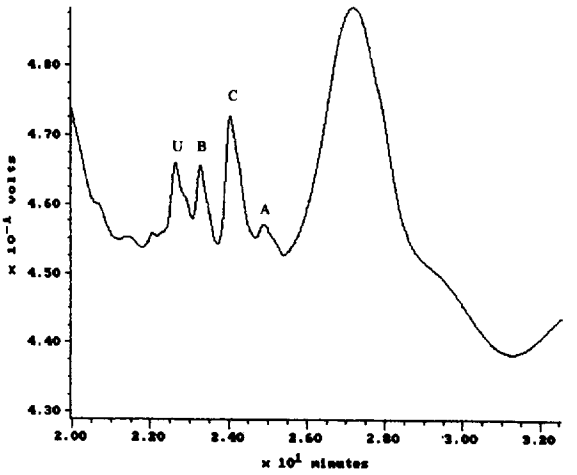

Fig. 8. Chromatogram of a 180-min saliva (application experiment) using the on-line IAC-RPC method. Injection volume: 4.5 ml of deproteinized and diluted (3:2, v/v) saliva. Calculated amounts were Δ^9-THC (A), 20.3 ng/ml; CBD (B), 23.3 ng/ml and CBN (C), 35.5 ng/ml. Unknown component (U) at 22.5 min. For conditions, see Section 2.

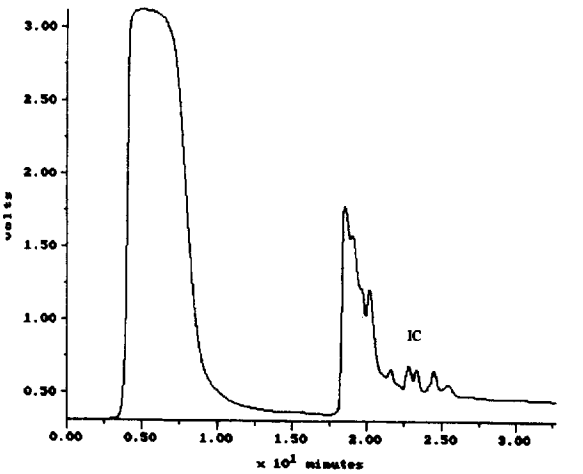

Fig. 9. Chromatogram of a blank saliva sample. Injection volume: 4.5 ml of deproteinized and diluted (3:2, v/v) saliva. The analytical column was switched in series (switching valve in position B) to the IAC column during the whole chromatographic run. IC, interfering components. For conditions, see Section 2.

Table 2
Recovery from blank saliva samples spiked with Δ^9-THC, CBD and CBN

Compound	Concentration (ng/ml)	Recovery (%)	Standard deviation (%)
Δ^9-THC	20	64.0	2.6
	30	65.6	2.3
	50	68.6	2.1
	100	71.0	1.7
	250	71.9	1.7
	500	73.8	1.4
	1000	73.9	1.4
CBD	10	65.9	2.6
	20	70.4	2.4
	30	69.7	2.2
	50	70.9	2.1
	100	71.0	2.0
	250	72.0	1.5
	500	73.7	1.4
	1000	75.2	1.3
CBN	10	72.8	2.6
	20	73.5	2.5
	30	75.1	2.3
	50	77.0	1.9
	100	77.0	1.7
	250	79.3	1.7
	500	81.4	1.5
	1000	83.2	1.3

Injection of 4.5 ml deproteinized saliva (dilution 3:2, v/v; $n = 3$). For chromatographic conditions, see Section 2.

profiles of spiked samples was observed. This included a decrease in the peak-area quotients CBD/CBN and Δ^9-THC/CBN, while the peak-area quotient of the fourth compound was raised. Table 6 summarizes the results of this experiment.

These results demonstrate that the method should

Table 3
Intra-day precisions at three concentration levels on saliva samples spiked with Δ^9-THC, CBD and CBN

Compound	Spiked concentration (ng/ml)	Found concentration (mean) (ng/ml)	S.D. (ng/ml)	C.V. (%)
Δ^9-THC	50	34.52	1.1	3.18
	100	70.55	1.33	1.89
	500	366.57	5.66	1.54
CBD	50	35.54	0.78	2.19
	100	71.24	1.27	1.78
	500	367.32	5.3	1.44
CBN	50	38.44	0.74	1.93
	100	77.1	1.41	1.83
	500	408.03	5.59	1.37

Injection of 4.5 ml deproteinized saliva (dilution 3:5, v/v; control group (CA); $n = 5$). For chromatographic conditions, see Section 2.

Table 4
Inter-day precisions at three concentration levels on saliva samples spiked with Δ^9-THC, CBD and CBN

Compound	Spiked concentration (ng/ml)	Found concentration (mean) (ng/ml)	S.D. (ng/ml)	C.V. (%)
Δ^9-THC	50	33.99	1.46	4.30
	100	70.07	2.39	3.41
	500	365.40	8.48	2.32
CBD	50	35.27	1.18	3.35
	100	70.98	2.18	3.07
	500	366.08	6.6	1.80
CBN	50	38.30	1.18	3.08
	100	76.75	2.23	2.91
	500	408.56	7.74	1.89

Injection of 4.5 ml deproteinized saliva (dilution 3:2, v/v; control group (CA); $n = 15$). For chromatographic conditions, see Section 2.

Table 5
Intra-system recovery and extra-system losses at three concentration levels of saliva samples spiked with Δ^9-THC, CBD and CBN

Compound	Concentration (ng/ml)	Recovery[a] (%)	S.D. (%)	Recovery (CB)−recovery (CA)[b] (%)
Δ^9-THC	50	71.5	1.6	2.4
	100	71.9	1.3	1.4
	500	73.9	0.9	0.6
CBD	50	87.1	1.0	16
	100	88.6	0.9	17.4
	500	89.3	0.8	15.9
CBN	50	79.1	1.2	2.2
	100	80	1.4	2.9
	500	81.9	0.8	0.3

Injection of 4.5 ml deproteinized saliva (dilution 3:2, v/v; control groups (CA) and (CB); $n = 5$). Intra-system extraction yield as recovery of control group (CB)[a] and calculated extra-system losses[b]. For chromatographic conditions, see Section 2.

Table 6
Δ^9-THC, CBD and CBN levels in saliva before and after the experimental oral administration of a cannabinoid mixture

Compound	Concentration (ng/ml)				Quotient (area *X*/area CBN)		
	Before	1 h	3 h	4 h	Typical	1 h	3 h
Δ^9-THC	n.q.	135.1	20.3	n.q.	0.38	0.28	0.21
CBD	n.q.	157.7	23.4	n.q.	0.57	0.50	0.37
CBN	n.q.	178.3	35.5	n.q.	1	1	1
U	–	p	p	–	–	0.09	0.60

A subject was given a cannabinoid standard mixture in ethyl alcohol, containing 5 mg each of Δ^9-THC, CBD and CBN. Samples were collected 1 h before administration and 1, 3 and 4 h after administration. Injection of 4.5 ml deproteinized saliva (dilution 3:2, v/v; $n = 2$). For chromatographic conditions, see Section 2. Typical quotients (area *X*/area CBN) from recovery experiments. U, unknown compound (see Fig. 8); n.q., below quantification limit; p, present.

be applicable for the identification of a present cannabis intoxication in law enforcement purposes.

4. Conclusion

The great advantage of the column-switching method described in this paper is the minimization of sample preparation steps through omission of tedious and non-selective pre-chromatographic extraction steps. Although one chromatographic run requires about 35 min, this assay may offer an attractive alternative to conventional chromatographic methods for Δ^9-THC analysis from saliva. However, the rapidity of the technique can be enhanced when alternative switching arrangements are used to facilitate multiple on-line sample extraction. The arrange-

ment used for our experiments was selected to monitor all chromatographic fractions of saliva. Further, the sensitivity of the assay can be increased when electrochemical detection is used. We selected UV detection to check the selectivity of the immunoaffinity enrichment.

Acknowledgments

We thank G. Moskopp (Supelco) and Mr. Senkfelder (Merck) for technical advice and sampling chemicals and equipment. We also express our appreciation to D. Bieniek (GSF, Oberschleissheim, Germany) and A. Conrad (Städtische Kliniken, Kassel, Germany) for helpful suggestions and critical comments.

References

[1] L. Grinspoon and J.B. Bakalar, Marihuana, The Forbidden Medicine, Yale University Press, New Haven, 1993.
[2] W. Dewey, Pharm. Rev., 38 (1986) 151.
[3] M. Perez-Reyez, W. White, S. Mc Donald, R. Hicks and A. Jeffcoat, Pharmacol. Biochem. Behav., 40 (1991) 691.
[4] L. Thompson and E. Cone, J. Chromatogr., 421 (1987) 91.
[5] C. Maseda, K. Hama, Y. Fukui, K. Matsubara, S. Takahashi and A. Akane, Forensic Sci. Int., 32 (1986) 259.
[6] D. Menkes, R. Howard, G. Spears and E. Cairns, Psychopharmacology, 103 (1991) 277.
[7] S. Kanter, L. Hollister and M. Williams, J. Chromatogr., 234 (1982) 255.
[8] P. Twitchett, P. Williams and A. Moffat, J. Chromatogr., 149 (1978) 683.
[9] J. Valentine and P. Psaltis, Anal. Lett., 12 (1979) 855.
[10] J. Vinson and A. Patel, J. Chromatogr., 307 (1984) 493.
[11] S. Kanter and L. Hollister, J. Chromatogr., 151 (1978) 225.
[12] S. Kanter, L. Hollister and M. Musumeci, J. Chromatogr., 234 (1982) 201.
[13] K. Kaistha and R. Tadrus, J. Chromatogr., 237 (1982) 528.
[14] G. Nakamura, W. Stall, V. Folen and R. Masters, J. Chromatogr., 264 (1983) 336.
[15] H. Hendriks, S. Batterman, R. Bos, H. Huizing and M. Malingré, J. Chromatogr., 205 (1981) 444.
[16] E. Garrett and C. Hunt, J. Pharm. Sci., 66 (1977) 20.
[17] P. Williams, A. Moffat and L. King, J. Chromatogr., 155 (1978) 273.
[18] P. Williams, A. Moffat and L. King, J. Chromatogr., 186 (1979) 595.
[19] H. Borys and R. Karler, J. Chromatogr., 205 (1981) 303.
[20] L. Karlsson, J. Chromatogr., 417 (1987) 309.
[21] E. Samara and M. Bialer, J. Chromatogr., 416 (1987) 370.
[22] Y. Nakahara and C. Cook, J. Chromatogr., 434 (1988) 247.
[23] P. Zweipfennig, J. Lisman, A. Van Haren, G. Dijkstra and J. Holthuis, J. Chromatogr., 456 (1988) 83.
[24] D. Fenimore, R. Freeman and P. Loy, Anal. Chem., 45 (1973) 2331.
[25] E. Bachmann, A. Hofmann and P. Waser, J. Chromatogr., 178 (1979) 320.
[26] A. Jones, M. Elsohly, J. Bedford and C. Turner, J. Chromatogr., 226 (1981) 99.
[27] K. Harzer and M. Kächele, J. Chromatogr., 278 (1983) 63.
[28] J. Rosenfeld, Y. Moharir and S. Sandler, Anal. Chem., 61 (1989) 925.
[29] D. Josic, W. Hofmann, R. Habermann, A. Becker and W. Reutter, J. Chromatogr., 397 (1987) 39.
[30] D. Deutsch and E. Mertz, Science, 170 (1970) 1095.
[31] H. Sternbach, Chromatographische Methoden in der Biochemie, G. Thieme Verlag, Stuttgart, 1991, p. 139.
[32] G. Georgiev, Y. Ilyin and A. Ryskov, Science, 195 (1977) 394.
[33] S. Roy, D. Weber and W. McGregor, J. Chromatogr., 303 (1984) 228.
[34] R. Glenncross, S. Abeywardene, S. Corney and H. Morris, J. Chromatogr., 223 (1981) 193.
[35] M. Sharman and J. Gilbert, J. Chromatogr., 543 (1991) 220.
[36] L. Reum, D. Haustein and J. Koolman, Z. Naturforsch., 36 (1981) 790.
[37] I. Tamir and D. Lichtenberg, J. Pharm. Sci., 72 (1983) 458.