Int J Legal Med (2004) 118: 326–331
DOI 10.1007/s00414-004-0469-9

ORIGINAL ARTICLE

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Development of a psilocin immunoassay for serum and blood samples

Received: 3 May 2004 / Accepted: 21 June 2004 / Published online: 3 August 2004

Abstract After the immunisation of rabbits with a psilocin-specific immunogen, polyclonal antisera were obtained. With these antisera a competitive, heterogeneous radioimmunoassay for the detection of psilocin was developed. As tracer a derivative of psilocin was synthesised, which contained a tritiated CH_3 group. The antisera showed a specific reaction with psilocin. The cross-reactivity of structurally related endogenous substances like serotonin, tryptophan and tyrosine was below 0.01%. Also common drugs of abuse (Δ^9-tetrahydrocannabinol, cocaine, morphine, amphetamine) showed negligible cross-reactivity (0.01–2%). Only tricyclic neuroleptics with a (dimethylamino)ethyl side-chain showed some cross-reactivity (20%). Spiked serum and blood samples were analysed with this new immunoassay and the results obtained were compared with the values measured with a validated GC-MS method.

Keywords Psilocin · Radioimmunoassay · GC-MS

Introduction

Fungi of the genus Psilocybe (e.g. *Psilocybe cubensis*, *Psilocybe mexicana*, *Psilocybe semilanceata*, so-called magic mushrooms) belong to the most frequently abused hallucinogenic drugs [1]. The hallucinogenic effects are caused by the compounds psilocybin and psilocin, indole derivatives with structural similarity to the neurotransmitter serotonin. Psilocybin represents a pro-drug and is rapidly cleaved to psilocin, the pharmacologically active agent [2, 3, 4, 5].

In many countries psilocybin and psilocin are classified as controlled substances, e.g. in the USA, Great Britain and Germany. The consumption of magic mushrooms causes various mental and physical disorders like retarded reactions, enlarged pupils and delayed pupillary reaction. Under these circumstances participation in road traffic can cause accidents and represent a risk. Therefore, it is necessary to analyse body fluids from suspected drivers to prove an abuse of drugs like psilocin. Different chromatographic methods have been described for the detection of psilocin in body fluids: REMEDi HS as a drug screening method based on HPLC [6], HPLC with electrochemical detection [5, 7, 8], GC-MS [6, 9], liquid chromatography with APCI-MS [10] and LC-MS-MS [11]. While the determination of psilocin in urine can be performed without sample clean-up, the analysis in plasma or serum requires an extraction procedure, followed by derivatisation of the extracts in the case of GC-MS.

Organised analytical procedures with immunological methods using antibodies which are either specific for a single substance or for a class of substances are very useful for drug screening [12, 13]. A result from an immunoassay can be differentiated into positive or negative and an estimation of the amount of drug may be possible. A positive result of an immunoassay has to be confirmed, e.g. with a chromatographic method.

Recently we have described the synthesis of an immunogen for psilocin (Fig. 1) [14]. With this immunogen we immunised five rabbits to raise antibodies against psilocin. A psilocin derivative with a tritiated methyl moiety at the indole nitrogen was synthesised as tracer (Fig. 2). After optimisation of the RIA procedure and evaluation of specificity and sensitivity, spiked serum and blood samples were analysed.

In addition a GC-MS method for psilocin after solid phase extraction was validated. The results of the RIA and the GC-MS method were compared.

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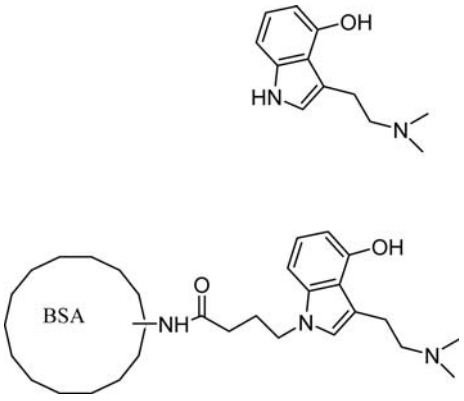

Fig. 1 Structural relationship of psilocin and the synthesised immunogen [15]

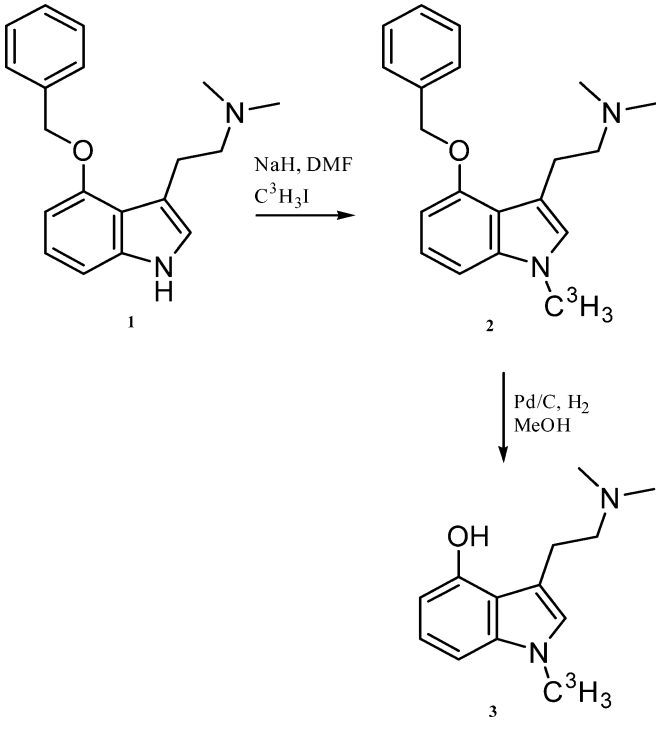

Fig. 2 Synthesis of a tritiated tracer for a radioimmunoassay

Material and methods

The measurements of radioactivity were performed on a Beckman LS 6000 LL ß-counter (Fullerton, USA).

Phosphate buffered saline (PBS, pH 7.4) for the dilution of antisera contained 8.0 g NaCl, 1.4 g $Na_2HPO_4 \times 2H_2O$, 0.2 g KH_2PO_4, 0.2 g KCl and 0.2 g NaN_3 per litre.

Phosphate buffer solution (0.1 M, pH 6.0) for the dilution of serum and blood samples consisted of 13.6 g KH_2PO_4/l H_2O adjusted to pH 6.0 by addition of 17.8 g $Na_2HPO_4 \times 2H_2O$/l H_2O.

GC was performed on an Agilent 6890 series gas chromatograph equipped with a HP 7683 series autosampler and a HP 5973 series mass selective detector.

Synthesis of tracers

[2-(4-Benzyloxy-1-[3H]-methylindole-3-yl)ethyl]dimethylamine 2 NaH (2.5 mg, 0.062 mmol, 60% dispersion in oil) was dissolved in dry dimethylformamide (DMF 200 µl) at 0°C and stirred for 15 min. The solution of 4-benzyloxy-*N,N*-dimethyltryptamine [15, 16] substance *1* (2.6 mg, 0.009 mmol) in dry DMF (300 µl) was added and the mixture stirred for further 20 min at 0°C. Subsequently a solution of [^{3}H]methyl iodide (85 mCi, 1.176×10^{-3} mmol, American Radiolabelled Chemicals, St. Louis, USA) in toluene (150 µl) was added and the resulting mixture was stirred overnight. Unreacted indole *1* was separated from radioactive indole *2* by thin layer chromatography (silica gel F_{254}, 10×20 cm, ethyl acetate/ethanol/triethylamine 84/12/2.8). The band with the product *2* was scratched off and eluted from the silica gel by stirring with ethyl acetate. After filtration the extraction procedure was repeated twice. The combined extracts were evaporated at 40°C under nitrogen to yield *2*.

3-(2-Dimethylaminoethyl)-1-[3H]-methyl-indol-4-ol 3 The radioactive indole *2* was dissolved in dry ethanol (300 µl), decanted into a hydrogenation flask and rinsed with hydrogen. A solution of Pd/C (11.8 mg/ml) in dry benzene (200 µl) was added and the mixture stirred under a balloon filled with H_2 for 20 min. The catalyst was removed by filtration through a cotton pad under nitrogen and the solvent evaporated under nitrogen.

Immunisation

Five rabbits were immunised at Dade Behring GmbH. The immunogen was dissolved in saline and mixed with Freund's complete adjuvant for subcutaneous (s.c.) injection and with aerosil for intravenous (i.v.) injections. The immunisation started with s.c. injection at days 1 and 14. From days 28–32 i.v injection was accomplished every day. The first antisera were recovered on day 36. The immunisation lasted a total of 141 days with blood being taken every 14 days and changing from i.v. to s.c. injection in the meantime.

Sample preparation for the immunoassay

For testing the antisera and for the determination of psilocin in serum and blood samples, a heterogeneous, competitive radioimmunoassay was chosen.

The tracer was diluted with PBS (pH 7.4) to a final activity of 2000 counts per minute (cpm) in 100 µl. For monitoring the immunisation, 100 µl tracer solution was incubated with 100 µl diluted antiserum (1:100, 1:250, 1:500) in a 1.5 ml vial for 75 min at room temperature. The solution was poured into a Vivaspin 2 ultra-filtration device (MWCO 50 kDa, Vivascience, Hannover, Germany), and the vial was rinsed 2 times with 200 µl PBS. The combined solution was centrifuged in an ultra-

filtration device (10 min at 2900 *g*). The filter was washed with 500 µl PBS (centrifugation 10 min at 2900 *g*). After addition of 200 µl of PBS to the ultra-filtration device, the antigen-antibody complex was recovered by reverse spinning (10 min at 2900 *g*). The separated radioactive complex was transferred in a scintillation vessel, the recovery cap of the ultra-filtration device rinsed 2 times with 150 µl PBS and the scintillation vessel filled with scintillation solution (Zinsser Analytic, Frankfurt, Germany). The vessels were then measured in the ß-counter.

To determine the sensitivity of the antisera 100 µl of a psilocin solution (Promochem, Wesel, Germany) in different concentrations (100 ng/ml, 50 ng/ml, 10 ng/ml) were mixed with 100 µl solution of the tracer (2000 cpm) and 100 µl diluted antiserum (1:100, 1:250, 1:500). Further sample treatment was as described above.

The specificity of the antiserum was determined with the 50% displacement method [17] whereby 100 µl of potent cross-reactants (10 ng/ml–10,000 ng/ml) was mixed with 100 µl tracer and 100 µl diluted antiserum (1:250) and treated as mentioned above.

For the determination of psilocin in serum and blood the radioimmunoassay was calibrated with aqueous solutions of psilocin in the range 10–1,000 ng/ml. The spiked serum samples (10 ng/g, 25 ng/g, 50 ng/g, 100 ng/g) were first centrifuged with regenerated cellulose filters (MWCO 30 kDa, Millipore, Bedford, Mass) and the filtrate was subsequently treated as described above. Blood (1 g) was diluted with PBS (1 ml) in a centrifuge glass tube and centrifuged, the supernatant transferred to regenerated cellulose filters, centrifuged and 100 µl filtrate was used for the radioimmunoassay.

GC-MS

For GC-MS 1 g of serum/blood was diluted with 1.5 ml phosphate buffer (pH 6.0, 0.1 M) 25 ng psilocin-d10 (Promochem, Wesel, Germany) in 25 µl methanol was added as internal standard, the samples centrifuged (10 min at 2900 *g*) and the supernatant used for solid phase extraction.

The Strata X columns (Phenomenex, Aschaffenburg, Germany) were conditioned with 1.5 ml methanol and 1.5 ml bidest. H_2O, the samples added and the columns washed 2 times with 750 µl of 5% methanol in H_2O. The columns were dried with a gentle stream of nitrogen (2 times for 20 s.). Sample elution was performed with methanol (2 times 750 µl) into a brown vial. The solution was evaporated to dryness under nitrogen at 40°C and the residue derivatised with 50 µl MSTFA solution (Fluka, Neu-Ulm, Germany) for 30 min at 70°C. The whole sample treatment was done without vacuum and light. The derivatives were separated on a J&W 128–5512 DB-5 MS fused-silica capillary column (12 m×0.2 mm i.d., 5% diphenyl 95% dimethylpolysiloxane with 0.33 µm film thickness). Instrument parameters were as follows: injection volume 2 µl, injector temperature 250°C, initial oven temperature 140°C held for 4 min then 10°C/min to 230°C held for 1 min, 25°C/min to 280°C held for 4 min, interface temperature 280°C, carrier gas helium, flow rate 1 ml/min. Qualitative and quantitative analyses were performed by comparison of retention times and relative abundance of three ions (psilocin-di-TMS 290.1, 348.1, 58.1) or two ions (psilocin-d10-di-TMS 292.1, 358.2) using the selected-ion monitoring (SIM) mode.

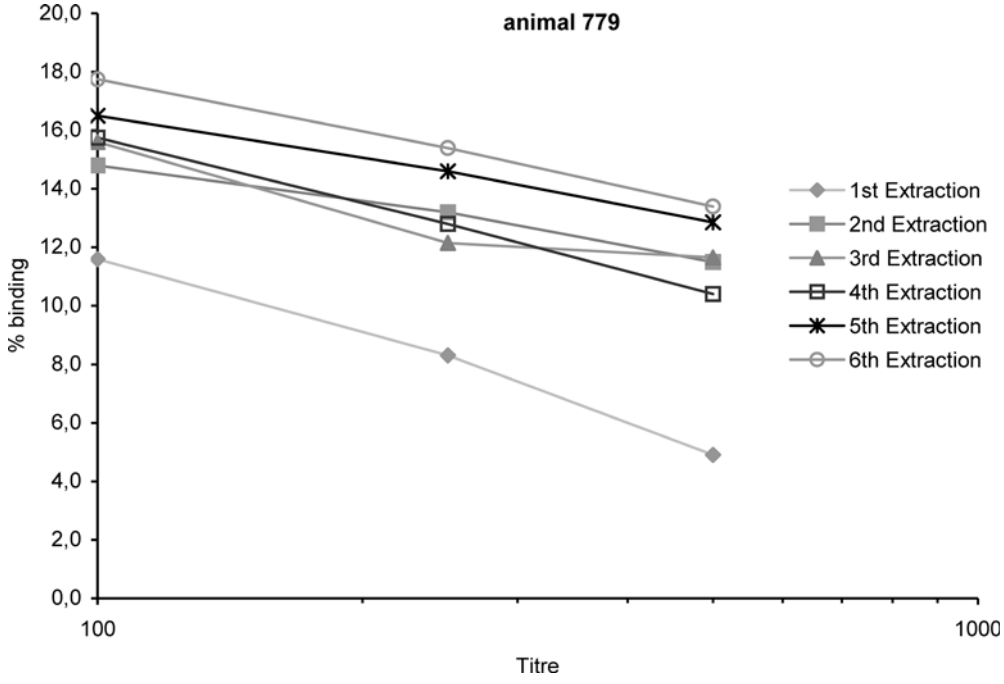

Fig. 3 Development of antiserum against psilocin, expressed as the ability of binding a tracer in relation to dilution of antiserum

Results and discussion

Synthesis

Synthesis of N-methylated psilocin was performed according to the method of Hofmann [18]. Since the amounts of the educts used in our case were very low (0.024 mol CH_3I in the synthesis by Hofmann versus 1.176×10^{-6} mol C^3H_3I in our synthesis), we had to optimise the reaction sequence. In the hydrogenation step a quantitative transformation of the educt to the product could not be achieved. Longer reaction times caused an increase in side-products, shorter reaction times produced a mixture of educt and the desired product (relation product/educt 70/30). Because the structure of the side-product and its reactivity with the antiserum was unknown, we decided to stop the hydrogenation after shorter reaction times although some educt *2* was present. Since the coupling of the synthesised hapten to the protein was carried out via a spacer at the indole nitrogen in position 1 (Fig. 1), we assumed that the characteristic structural features of psilocin, the phenolic OH-group and the (dimethylamino)ethyl side chain in position 3 of the indole ring system, are part of the epitope region and accessible for antibody generation. The educt *2* does not have a phenolic OH-group and therefore should not react as an antigen.

Immunisation monitoring

Several blood samples were taken during the immunisation process. To control the production and development of specific antiserum, the ability for binding radiolabelled tracer was determined. The antisera were diluted in the range from 1:100 to 1:500 and then incubated with a constant amount of tracer. The antigen-antibody complex was separated by filtration and the amount of bound tracer was measured and calculated as a percentage of the origin tracer amount after subtraction of non-specific binding. In Fig. 3 the immunisation progress is shown for one rabbit (no. 779). After 141 days the whole blood of the rabbits was collected because no further improvement in antiserum activity could be observed.

Sensitivity

In order to obtain a sensitive immunological test system, the ratio of the antibody binding sites and the tracer amount must be optimised. A constant amount of tracer was used to determine the antiserum dilution at which the addition of 1 ng psilocin results in a displacement of the tracer from the antibody binding sites. As shown in Fig. 4 an amount of 1 ng psilocin resulted in a distinct decrease of the tracer signal at a dilution of 1:250 and 4 out of the 5 immunised rabbits showed these results.

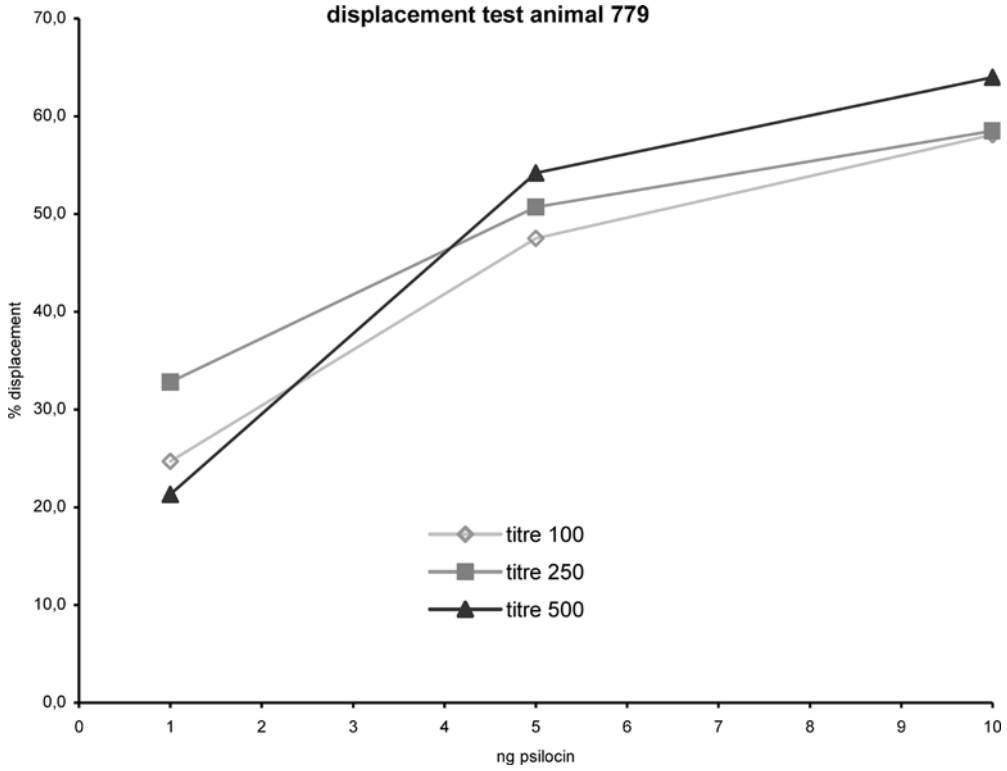

Fig. 4 Results of displacement test for antiserum 779 after addition of psilocin

Table 1 Cross-reactivity (%) of structurally related endogenous substances and drugs, common drugs of abuse and other drugs

Potential cross-reactant	Cross-reactivity
Structurally related endogenous substances and drugs	(%)
Serotonin	<0.01
Tryptophan	<0.01
Tryptamine	<0.01
Risperidon	<0.01
Indomethacine	<0.01
Sumatriptane	<2.0
Common drugs of abuse	
Δ^9-Tetrahydrocannabinol	<0.01
Δ^9-Tetrahydrocannabinol carboxylic acid	<0.01
Cocaine	<0.01
Benzoylecgonine	<0.01
Morphine	<0.01
LSD	<0.01
Mescaline	<0.01
6-Monoacetylmorphine	<2.0
Amphetamine	<2.0
3,4-Methylendioxy-*N*-methylamphetamine	<2.0
Diazepam	<2.0
Other drugs	
Ambroxol	<0.01
Promazine	<20
Levomepromazine	<20

Psilocin, 6-acetylmorphine (MAM), benzoylecgonine, cocaine, 3,4-methylendioxy-N-methylamphetamine (MDMA), Δ9-tetrahydrocannabinol, Δ9-tetrahydrocannabinol carboxylic acid, and Psilocin-d10 were obtained from Promochem, Wesel, Germany.
Amphetamine sulphate, diazepam, methadone hydrochloride, risperidon, serotonin sulphate and tryptamine were obtained from Sigma-Aldrich, Deisenhofen, Germany.
Mescaline, morphine hydrochloride×3H_2O and tryptophan were obtained from Merck, Darmstadt, Germany.
Levomepromazine and promazine were obtained from Bayer, Leverkusen, Germany.
Ambroxol hydrochloride was obtained from Dr. Karl Thomae, Biberach, Germany.
Indomethacine was obtained from Caesar and Lorentz, Hilden, Germany.
Lysergic acid dimethylamide was obtained from Sandoz, Basel, Switzerland.
Sumatriptane succinate was obtained from AApin Chemicals, Abingdon, UK.
Psilocin was employed in a concentration range 1–1,000 ng/ml, cross-reactants in the range 10–10,000 ng/ml.

Cross-reactivity

For further investigations the antiserum from animal 779 was chosen because it showed the highest decrease of the tracer signal at a dilution of 1:250. The evaluation of the specificity was ascertained with the 50% displacement method [17]. As potential cross-reactants, structurally related endogenous substances like serotonin, tryptamine and tryptophan and structurally related drugs like sumatriptane were chosen. In addition, common drugs of abuse were tested.

The structurally related endogenous substances and drugs showed negligible cross-reactivity (<0.01%–<2.0%). These results confirmed the chosen synthesis strategy for the immunogen. The characteristic structural features of the indole derivative psilocin, the (dimethylamino)ethyl side-chain in position 3 and the phenolic hydroxyl group in position 4, seem to be accessible as an epitope for antibody generation. This could be the reason for the negligible cross-reactivity of the indole serotonin, a neurotransmitter with a aminoethyl side-chain in position 3 and a phenolic hydroxyl group in position 5, and the other structurally related substances examined.

Also the common drugs of abuse tested (Table 1) showed very low cross-reactivity. Only the phenothiazine derivatives promazine and levomepromazine showed significant levels of cross-reactivity of 20% (Table 1) which may be attributed to the (dimethylamino)ethyl side-chain. This side-chain showed a high structural similarity to the side-chain of psilocin which is responsible for the generation of specific antibodies.

GC-MS validation

The GC-MS method for the determination of psilocin was validated with spiked serum samples. The calibration range was 5–200 ng/g and the linearity within this range was sufficient with a coefficient of correlation for the linear regression of R^2>0.999. Recovery of psilocin after addition of 10, 25 and 100 ng/g was 88%, the limit of quantitation (LOQ) and limit of detection (LOD) were determined corresponding to the guidelines of the U.S. Department of Health and Human Services [19]. The LOQ was 5 ng/g and the LOD was 3 ng/g.

Serum and blood samples

For the immunological determination of psilocin in serum and blood a calibration curve was recorded in the range 10–1,000 ng/ml with aqueous solutions. The linearity was assessed by half logarithm evaluation (R^2>0.999). For the direct measurement of spiked serum samples, proteins had to be separated by ultra-filtration with regenerated cellulose filters (MWCO 30 kDa). Reproducible results were obtained in the range 10–100 ng/g serum (Table 2). The lower results of RIA determination in comparison with GC-MS results may be caused by an unspecific binding of psilocin to serum proteins. The unspecific binding caused some psilocin to be retained in the residue on the filter. For this reason the filtrate used for the immunoassay contained a lower amount of psilocin than the serum sample prior to filtration.

The analytical methods for the determination of psilocin were used to analyse four blood samples from real cases, one heart blood sample of a deceased (cause of death: multiple trauma) and three venous blood samples of

Table 2 Comparison of GC-MS and RIA results for determination of psilocin in spiked serum samples

Spiked concentration psilocin	GC-MS	RIA
(ng/g)	(ng/g)	(ng/g)
10	10.6	5
25	23.6	14
100	98.2	68

Table 3 Results for the RIA detection of psilocin in case blood samples

Case samples	RIA (ng/g)
Case 1 (deceased)	50
Case 2 (traffic case)	n.d.
Case 3 (traffic case)	38
Case 4 (traffic case)	8

n.d. Not detectable.

suspected criminal offenders. By GC-MS psilocin could not be detected. These results indicate that the extraction procedure does not provide sufficient extracts from blood samples for the GC-MS analysis. Although the current literature refers to the analysis of plasma or serum samples, the analysis of blood has not yet been described. In contrast the RIA showed a positive result in three out of four samples (Table 3).

Conclusions

For the first time a specific immunoassay for the detection of the hallucinogen psilocin in body fluids is described. The RIA procedure is a specific immunological method for the determination of psilocin in serum or blood samples. Only short sample pre-treatment was needed and an estimation of the amount of psilocin is possible. The main advantage is the possibility to analyse whole blood samples.

Acknowledgement The authors wish to thank Dr. W. Schneider and the staff from Dade Behring GmbH Marburg who were responsible for the animal experimental section.

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