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Quantitation of psilocin in human plasma by high-performance liquid chromatography and electrochemical detection: comparison of liquid–liquid extraction with automated on-line solid-phase extraction

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Abstract

Two modifications of the HPLC–ED method with respect to extraction procedure used have been developed for psilocin, the active metabolite of psilocybin, in human plasma using either liquid–liquid extraction (LLE) or automated on-line solid-phase extraction (on-line SPE). Each type of the sample preparation required a different HPLC system followed by electrochemical detection at 650 to 675 mV. The limit of quantitation of both modifications was 10 ng/ml psilocin. There was no significant difference observable between the LLE and the on-line SPE in terms of method standard deviation (LLE 1.82%, on-line SPE 1.13%) and the analytical results. However, the advantages of on-line SPE in addition to different selectivity were less manual effort, smaller plasma volumes of 400 μl (LLE 2 ml) and a recovery of psilocin in human plasma of nearly 100% (LLE 88%). In contrast to a previous procedure both methods were rapid, simple and reliable and yielded high plasma recoveries. They were used successfully in the quantitation of psilocin in plasma samples obtained from healthy volunteers after p.o. administration of 0.2 mg psilocybin per kg body mass. Plasma concentration curves and pharmacokinetic parameters were calculated.

Keywords: Psilocin; Solid-phase extraction; Liquid–liquid extraction

1. Introduction

The hallucinogen psilocybin, whose effect is mediated via 5-HT_2 receptors [1], was isolated from the Mexican hallucinogenic mushroom *Psilocybe mexicana* and then synthesized by Hofmann et al. in 1959 [2]. The quantitation of psilocybin and its biosynthesis precursor psilocin was first carried out in fungal material primarily using high-performance liquid chromatography (HPLC) with UV, fluorescence or electrochemical detection (ED) as analytical method [3–6]. The metabolism of psilocybin was first investigated by paper chromatography [7,8] and HPLC [9] in rats and later by gas chromatography (GC) and HPLC in humans [10], whereby psilocin together with 4-hydroxyindol-3-ylacetic acid and 4-hydroxytryptophol were identified as metabolites. Psilocin is already produced from psilocybin in the

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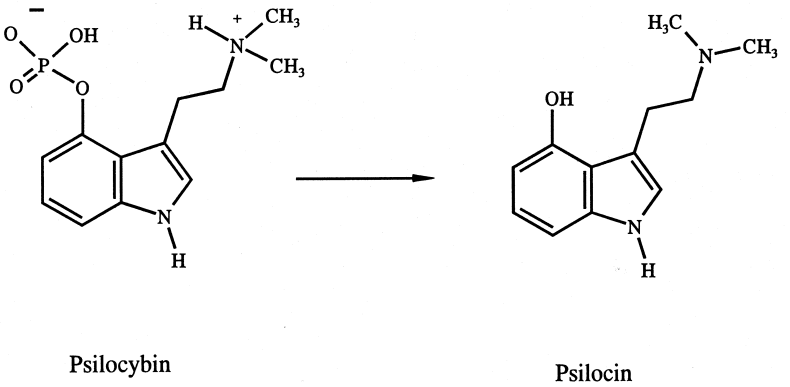

Fig. 1. Metabolism of psilocybin in the gut.

gut as a result of dephosphorylation by alkaline phosphatase and is, thus, the actual active substance (Fig. 1). Psilocin is an easily oxidizable substance. Therefore liquid–liquid extraction (LLE), on-line solid-phase extraction (SPE) [11] and column switching methods [12] are appropriate for sample preparation. Psilocin concentrations in plasma have been investigated using in vitro microdialysis to separate proteins from plasma followed by column switching HPLC with ED [12]. The in vitro microdialysis leads to a low recovery rate of 15%, a high plasma requirement of 3 ml and a sample preparation time of 12 h. Summing up, this method is not a convincing one. This paper, therefore, describes two rapid, robust and reproducible HPLC procedures with electrochemical detection, that allow quantitative determination of psilocin in plasma. In one the sample preparation employs LLE and in the other on-line SPE. Plasma concentration curves have been constructed using plasma samples obtained from seven healthy volunteers after p.o. administration of 0.2 mg psilocybin per kg body mass and pharmacokinetic parameters have been calculated.

2. Experimental

2.1. Materials

Psilocybin and psilocin-base were synthesized according to Refs. [2,13,14]. The internal standards bufotenine monooxalate·H_2O and 5-hydroxyindole were obtained from Sigma (St. Louis, MO, USA). Citric acid, ethylendiamine-tetraacetic acid disodium salt dihydrate, lithium acetate, sodium acetate and potassium dihydrogenphosphate were of analytical grade (Fluka, Buchs, Switzerland). Acetic acid analytical-reagent grade, dichloromethane LiChrosolv, sodium carbonate analytical-reagent grade and orthophosphoric acid 85% analytical-reagent grade were supplied by Merck (Darmstadt, Germany). Methanol and acetonitrile were purchased from Rathburn (Zinsser Analytic, Frankfurt, Germany) and poly(ethylene glycol) (PEG) 6000 in DAB quality from Merck–Schuchardt (Hohenbrunn, Germany).

Water was deionized and twice distilled. Human plasma for reference was obtained from a blood bank (University Clinic, Tübingen, Germany). The real plasma samples were provided by a clinical study (Department of Psychiatry and Psychotherapy, Technical University of Aachen, Germany). Plasma was kept under −20°C before analysis.

OSP-2a cartridges 10 mm×4 mm (Merck) were loaded, dosed by volume, with CBA cation-exchange sorbent (functional group carboxymethyl) of the particle size 60 μm (ICT, Frankfurt, Germany). For LLE Extrelut 3 columns were used (Merck).

In both modifications the LC was carried out on a LiChroCart Superspher 60 RP-select B column, 5 μm, 250×4 mm I.D. using a guard column LiChrospher 60 RP-select B, 5 μm, 4×4 mm I.D. (Merck).

2.2. Equipment

The HPLC station comprised an isocratic HP 1050 pumping system, a HP 1050 autosampler with a 1000-μl injection loop, a HP 1049 programmable electrochemical detector, a HP 3396 Series II integrator and a HP 9114 B disk drive all from Hewlett-Packard (Waldbronn, Germany). Additional the on-line SPE used a gradient pump L-6200 A, an autosampler L-7200 both from Merck–Hitachi (Darmstadt, Germany) and an OSP-2 sample preparator from Merck.

2.3. Sample preparation

2.3.1. Liquid–liquid extraction

Aqueous psilocin and methanolic 5-hydroxyindole standard solutions were always made up freshly as required. Aliquots of 2 ml plasma were adjusted to a pH of 8.5 with 50 μl of 0.5 molar sodium carbonate solution. Protein was precipitated by adding 1.2 ml methanol, in which 10 ng 5-hydroxyindole as internal standard were solved. The precipitated proteins were centrifuged off (2875 *g*/10 min/20°C). The supernatant was applied to an Extrelut 3 column, which was eluted with two portions of 6 ml dichloromethane. The eluate was concentrated to dryness at 40°C in a stream of nitrogen and taken up in 100 μl dichloromethane of which 10 μl was injected into the HPLC system.

2.3.2. Automated on-line, solid-phase extraction

Stock solutions were prepared by dissolution of psilocin and bufotenine in methanol; these were then freshly diluted in water to yield aqueous standard solutions. The stock solutions were stored at −15°C. Real plasma samples were analysed by adding 20 μl internal standard (120 ng bufotenine/ml) to 400 μl freshly thawed plasma and precipitating the plasma proteins with 400 μl PEG 6000 solution 20% under cooling in an ice bath (5 min). Then the samples were centrifuged at 2875 *g*/3 min/20°C. Aliquots of 410 μl (corresponding to 200 μl plasma) were applied to conditioned CBA cartridges by means of an autosampler (see Tables 1 and 2 for details).

2.4. HPLC conditions and detection

2.4.1. LLE method

A LiChroCart, Superspher 60 RP select B column, 5 μm, 250×4 mm was used for separation. The mobile phase consisted of aqueous buffer solution (0.1 *M* sodium acetate, 0.1 *M* citric acid, 0.03 m*M* Na_2EDTA, pH 4.1)–acetonitrile (83:17, v/v). The flow-rate was 700 μl/min and the electrochemical detection was carried out at a potential of +650 mV.

2.4.2. On-line SPE method

The separation was carried out isocratically on a LiChroCart, Superspher 60 RP select B column, 5 μm, 250×4 mm. The mobile phase consisted of 150

Table 1
Processing scheme for psilocin in human plasma

LLE method	On-line SPE method
Pipette 2 ml plasma	Pipette 400 μl plasma
Add 1.2 ml methanol, including $I.S._1$ 10 ng	Add 20 μl $I.S._2$ solution (2.4 ng)
Add 50 μl 0.5 *M* Na_2CO_3 until pH 8.5	Add 400 μl PEG 6000 solution 20%
Cool 5 min (ice-bath)	Cool 5 min (ice-bath)
Centrifuge at 2875 *g*/10 min/20°C	Centrifuge at 2875 *g*/3 min/20°C
Give supernatant on column	
Wait 5 min	
Elute with 6 ml CH_2Cl_2 (5 min)	
Elute a second time with 6 ml CH_2Cl_2 (10 min)	
Evaporate under nitrogen, 40°C	
Add 100 μl CH_2Cl_2	Load vials into autosampler rack
Inject 10 μl	Inject 410 μl

$I.S._1$=5-hydroxyindole, $I.S._2$=bufotenine.

Table 2
Time program for the on-line SPE method

Time	% A	% B	% C	Valve 1	Valve 2	Clamp	Move	Flow	Remarks
0	0	0	100	On	Off	Closed	Off	0.5	Injection and washing of sample
6.0	0	0	100	Off	Off	Open	Off	0.5	Clamp open
6.1	0	0	100	Off	Off	Open	On	0.5	Ring moves one position
6.2	0	0	100	Off	Off	Closed	Off	0.5	Clamp close
6.3	100	0	0	Off	On	Closed	Off	2.0	Start of elution onto the analytical column, start Integrator
8.3	100	0	0	On	Off	Closed	Off	2.0	End of elution, activation of new cartridge
9.8	100	0	0	Off	Off	Closed	Off	2.0	
9.9	0	100	0	Off	Off	Closed	Off	2.0	
11.9	0	100	0	Off	Off	Closed	Off	2.0	
12.0	0	100	0	On	Off	Closed	Off	2.0	Washing of cartridges before re-use
22.0	0	100	0	Off	Off	Closed	Off	2.0	
22.1	0	0	100	Off	Off	Closed	Off	2.0	
24.1	0	0	100	Off	Off	Closed	Off	2.0	
24.2	0	0	100	On	Off	Closed	Off	2.5	Pre-conditioning of cartridge
29.2	0	0	20	On	Off	Closed	Off	2.5	

A: Methanol, B: acetic acid, C: lithium acetate solution, pH 6.8; flow in ml/min, valve 1: sample enrichment, valve 2: elution.

mmol potassium dihydrogenphosphate buffer pH 2.3–acetonitrile (94.5:5.5, v/v) with 160 μmol Na_2EDTA in the buffer–acetonitrile mixture. The flow-rate was 600 μl/min. The potential of the electrochemical detector was set at +675 mV.

2.5. Validation

For each method equidistant calibration standard points were measured in two different ways: (a) directly in aqueous and (b) after sample pretreatment from spiked plasma solutions. Therefore the recovery could be calculated over the whole working range. In addition ten spiked plasma concentrations of the high working range as well as of the low working range were determined. The homogeneity of variance, the precision and the accuracy were calculated by the received data [15].

2.6. Study design and sampling

Oral doses of 0.2 mg psilocybin per kg body weight, but maximum 15 mg per person were administered in a placebo-controlled, double-blind study with randomized allocation of psilocybin to seven healthy volunteers (self-experimentation by physicians). This study has been approved by the ethical committee of the department of medicine in Aachen, Germany. In order to study pharmacokinetics, blood samples were taken at −15, 0, 15, 30, 50, 70, 90, 110, 140, 180, 240, 300, 360 and sometimes 420 min after administration. The blood samples were centrifuged in heparinized tubes at 2875 *g*/10 min/20°C, the supernatant plasma was then deep frozen at −20°C and analysed on the following day by HPLC–ED.

3. Results and discussion

3.1. Analytical procedures

During development of the method it was found that conventional off-line SPE was unsuitable, since the easily oxidized psilocin decomposed during the drying process in the extraction procedure. The decomposition takes place because of the interaction between the extensive surface of the sorbent in the SPE columns, psilocin and air. Therefore this conventional extraction procedure led to unacceptable recoveries which were lower than 50% and showed great variety. In contrast off-line LLE is a more gentle procedure. So that this method (LLE method) was selected for sample preparation. By varying the pH of the aqueous phase from 8 to 9 no decrease of recovery could be observed. A RP select B column

was used as stationary phase and to the mobile phase a small quantity of Na_2EDTA was added to complex interfering ions. Detection was carried out electrochemically, because UV detection was too insensitive. After determination of hydrodynamic voltammograms for psilocin and 5-hydroxyindole (internal standard) the oxidation potential was set at +650 mV, in the region of the limiting current, so that small variations in the potential did not influence the signal.

It was possible to considerably simplify and, as it turned out, improve the determination of psilocin by the use of an automated on-line sample preparation employing the OSP 2 sample preparator of Merck [16] (on-line SPE method). It was necessary to adapt the parameters for liquid chromatographic separation and electrochemical detection to the requirements of the CBA sorbent (reported pK_a between 4.2 and 4.8) used for on-line SPE. For this reason the flow-rate of the mobile phase was reduced from 700 μl/min to 600 μl/min and bufotenine instead of 5-hydroxyindole was selected as internal standard for the on-line SPE. Bufotenine only differs from psilocin in the position of the hydroxy group (position 5) at the indole ring. Taking account of the hydrodynamic voltammogram of bufotenine the oxidation potential was increased to +675 mV. The variations in on-line SPE as a result of differences in the extraction cartridges were negligible, as was the peak broadening resulting from on-line coupling. The extraction cartridges could be re-used at least five times. Besides of the protein denaturation step the on-line SPE method was fully automated. Possible variations in analytical results caused by different operators were minimized.

Supposing parallel work-up the mean analysis time was 30 min for each modification. But the amount of manual work involved for the laboratory staff is appreciably less in the case of on-line SPE.

Probably the greatest advantage of on-line SPE is the low plasma requirement. Only 400 μl plasma are sufficient to reach a limit of quantitation of 10 ng/ml (defined by 95% confidence interval of the calibration curve [17]). Five times the volume (2 ml) is required to reach the same determination limit by LLE (cf. Table 1).

The comparability of the LLE and on-line SPE has been checked on the basis of statistical parameters (see Tables 3 and 4). They were found to be comparable with respect to precision, accuracy and method standard deviation. A Student *t*-test was carried out to compare the recoveries (means over the whole working range). Calculation revealed $t=6.79$ compared with the tabulated $t=2.20$ for $P=95\%$, leading to rejection of the zero hypothesis. This means that on-line SPE yielded significantly superior recovery. The HPLC chromatograms in Fig. 2 confirm the high and differing selectivities of the two methods. Psilocin (1) was identified in B and D by comparison with blank plasma (A, C) and spiked plasma samples (chromatograms not shown).

Comparison with the microdialysis method described in the literature [12] reveals that both LLE and on-line SPE exhibit appreciably better recovery,

Table 3
Comparison of statistical parameters

	LLE method	On-line SPE method
Working range	200–5800 pg	250–5250 pg
Number of calibration standard points	8	5
Coefficient of correlation	0.9997	0.9999
Recovery (whole working range)	88%	100%
Standard deviation	2.7%	3.5%
Variance	$7.4\%^2$	$12.3\%^2$
Relative standard deviation	3.1%	3.5%
Homogeneity of variance	9.53	6.58
	$P=99.9\%$ performed	$P=99.9\%$ performed
Precision high working range ($N=10$)	1.4%	1.3%
Precision low working range ($N=10$)	11.4%	11.0%
Accuracy high working range ($N=10$)	+4.9%	+1.8%
Accuracy low working range ($N=10$)	−3.9%	+5.6%

Table 4
Parameters of calibration

	a_0	a_1	s_y	s_{x0}	V_{x0}
LLE method	$-5.7817 \cdot 10^4$	1497.2	$8.7181 \cdot 10^4$	58.23	1.82
On-line SPE	$-6.8271 \cdot 10^4$	1865.4	$5.8113 \cdot 10^4$	31.15	1.13

$y = a_0 + a_1 x$.
s_y = Residual standard deviation; s_{x0} = method standard deviation and V_{x0} = relative method standard deviation.

a higher precision, a considerably lower plasma requirement and the analysis time is reduced at least twelve-fold. Also, in contrast to the microdialysis method, the methods presented here employ an internal standard in order to increase the robustness and a potential in the range of the limiting current instead of at a potential of 150 mV.

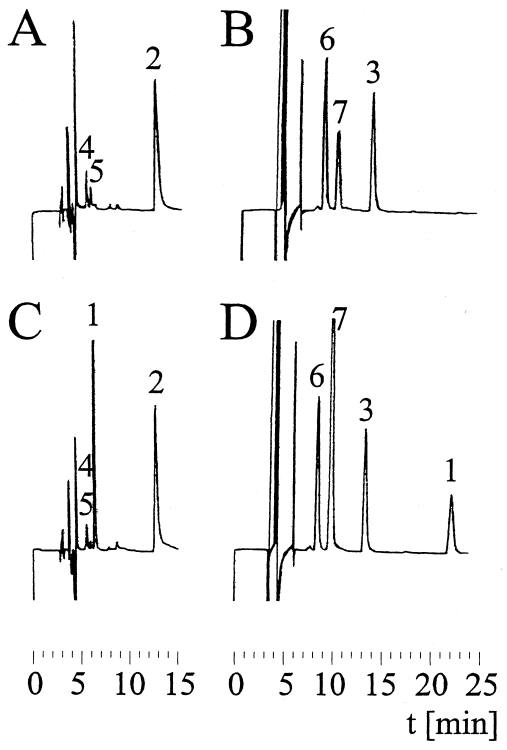

Fig. 2. HPLC chromatograms obtained from real plasma samples of volunteer 6 after 0 min (A, B) and 70 min (C, D) after p.o. administration of 0.2 mg/kg psilocybin. (A, C) LLE method; (B, D) on-line SPE method. 1=Psilocin; 2=5-hydroxyindole (I.S., LLE), 3=bufotenine (I.S., on-line SPE, 4, 5, 6, 7=biomolecules. Retention times in (C): 1=6.71 min, 2=13.62 min, 4=6.00 min, 5=6.18 min. Retention times in (D): 1=23.07 min, 3=13.86, 6=8.73, 7=10.13 min.

3.2. Comparison of the analytical results

In order to establish that the LLE and the on-line SPE lead to the same analytical results the plasma samples from volunteer 6 in the clinical study already mentioned (cf. Section 2.5) were analysed using both procedures (Fig. 3). The plasma concentration curves, obtained with both methods, had analogous shapes. In both cases it was only possible to quantify psilocin in plasma after 50 min. The mean maximum plasma concentration was 6.0 ng/ml psilocin after 90 min. The curve then fell and after 360 min the mean plasma concentration was 3.2 ng/ml psilocin. The correlation coefficient, calculated to compare the analysis results of LLE and on-line SPE, was found to be 0.995. The table value for 99.9% probability of $r = 0.772$ is far below the calculated value so that the analysis results are related. Furthermore, a Wilcoxon test for pair differences [18] was carried out to provide a statistical analysis of paired random samples; this test is suitable for random samples such as these that are not normally distributed. The value obtained for the test parameter R was 3.0, which is smaller than the tabulated R value of 5.0 ($n = 9$, differences of 0 were not taken into account). Hence, the zero hypothesis is fulfilled and the analysis results from the two modifications are equivalent. To summarize it has been demonstrated that these procedures do not differ statistically significantly with respect to the method standard deviation or the results of analysis. LLE and on-line SPE lead to the same results with the same probability.

3.3. Plasma concentration curves and pharmacokinetic parameters

LLE and the on-line SPE described above have been used to determine the psilocin concentrations in

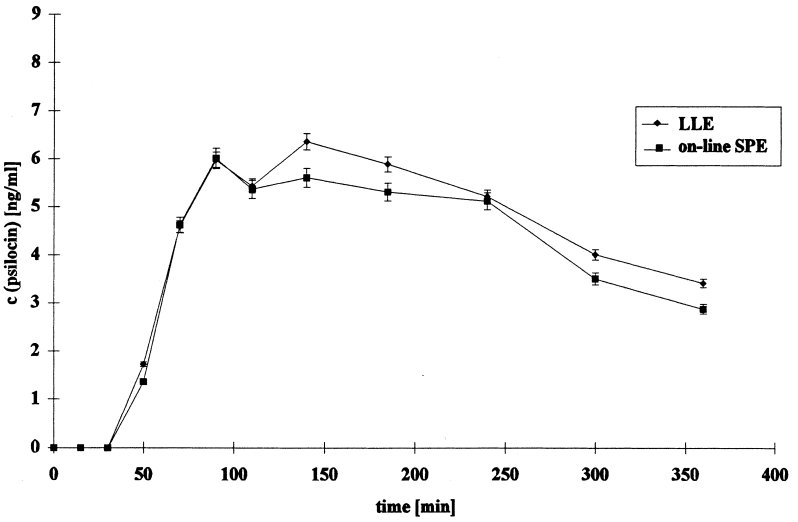

Fig. 3. Plasma concentration curves of psilocin (ng/ml) of volunteer 6 after p.o. administration of 10 mg psilocybin, comparison LLE with on-line SPE. Error bars: method standard deviation.

the plasma samples obtained in the clinical study and to construct the plasma concentration curves (Fig. 4). The LLE, which was developed first, was used for extraction. This was not possible for the samples from volunteer 5 since a plasma component overlapped with psilocin. For this reason extraction was carried out in this case by means of on-line SPE, which proved suitable on account of its differing selectivity. The samples from volunteer 6 were measured, as described in Section 3.2, by both the LLE and on-line SPE methods, so that here the mean results have been used to calculate the pharmacokinetic parameters (Table 5, Fig. 3). The time delay, until psilocin could be determined in the plasma, ranged from 15 to 50 min. Psilocin was still present in the plasma of all volunteers after 360 min; at 420 min there was no measurable concentration in the plasma of volunteer 5. In the case of volunteer 3

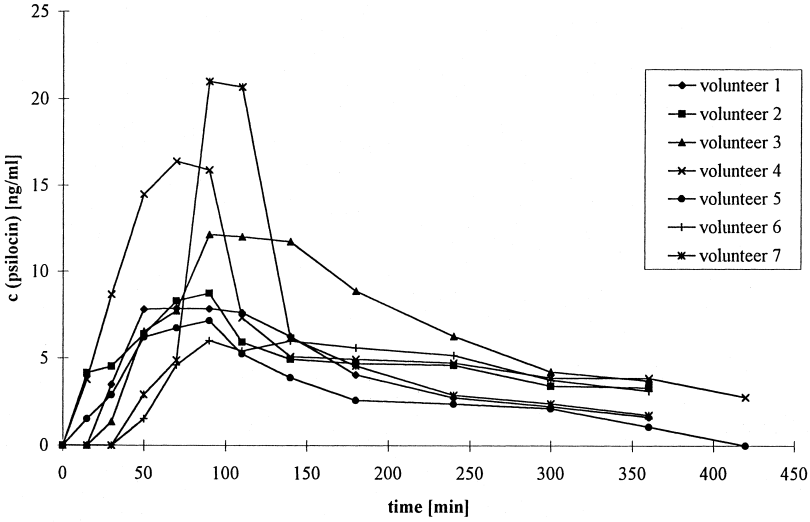

Fig. 4. Plasma concentration curves of psilocin (ng/ml) of volunteers 1, 2, 3, 4, 5, 6 and 7 after p.o. administration of 0.2 mg/kg psilocybin.

Table 5
Pharmacokinetic parameters

	P1	P2	P3	P4	P5	P6	P7
c_{max} (ng/ml)	7.9	8.8	12.1	16.4	7.2	6.0	21.0
t_{max} (min)	70	90	90	70	90	70	90
AUC (ng h/ml)	24.9	29.1	40.8	39.8	20.2	25.0	31.8

a plasma sample was taken on the day after the investigation, in which it was not possible to quantify any psilocin. The maximum plasma concentration (c_{max}) was between 6.0 (MIN value) and 21.0 ng/ml psilocin (MAX value) with a relative standard deviation (R.S.D.) of 48.9%, the time of maximum plasma concentration (t_{max}) was between 70 and 90 min (R.S.D.=11.6%) and the area under the curve (AUC), as calculated by the trapezoidal rule, was between 20.2 and 40.8 ng h/ml (R.S.D.=25.7%). These results reveal a large inter-individual variation, whereby the AUC is subject to appreciably less variation than c_{max}.

The one-compartment model used as the pharmacokinetic model yields an elimination rate constant (k_e) for psilocin of 0.307/h, a half life ($t_{1/2}$) of 2 h 15 min and an absorption rate constant (k_a) of 1.307/h.

The goodness of fit was proved by calculating the correlation coefficient (r) (P=95%) between the results predicted by the one-compartment model and the observed concentrations [19].

4. Conclusions

Psilocin can be extracted from human plasma by both the LLE and the one-line SPE method and quantitated by HPLC–ED. In contrast to the recently published method [12] both the procedures described are characterized by a high recovery rate, high selectivity and robustness and low time requirement. Using real samples very similar concentration profiles of psilocin in plasma could be detected by both modifications. The initially developed LLE method was used for most of the measurement since less complex apparatus is required. However, on-line SPE is to be preferred on account of the higher recovery rate for psilocin, the lower plasma consumption and the less demands on staff time. Two validated analytical methods with differing selectivities are a great advantage for analysis of biological materials, where various endogenous components are to be expected, which can be subject to inter-individual variation (cf. Section 3.3, volunteer 5).

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References

[1] O. Benkert and H. Hippius, Psychiatrische Pharmakotherapie, Springer Verlag, Berlin, 1992.
[2] A. Hofmann, R. Heim, A. Brack, H. Kobel, A. Frey, H. Ott, T. Petrzilka, F. Troxler, Helv. Chim. Acta 42 (1959) 1557.
[3] R. Kysilka, M. Wurst, J. Chromatogr. 320 (1985) 414.
[4] S. Borner, R. Brenneisen, J. Chromatogr. 408 (1987) 402.
[5] A.L. Christiansen, K.E. Rasmussen, J. Chromatogr. 210 (1981) 163.
[6] R. Vanhaelen-Fastré, M. Vanhaelen, J. Chromatogr. 312 (1984) 467.
[7] P.K. Gessner, P.A. Khairallah, W.M. McIsaac, L.H. Page, J. Pharmacol. Exp. Ther. 130 (1960) 126.
[8] F. Kalberer, W. Kreis, J. Rutschmann, Biochem. Pharmacol. 11 (1962) 261.
[9] R. Kysilka, J. Chromatogr. 534 (1990) 287.
[10] P. Holzmann and K.-A. Kovar, in Merck KGaA, Chromatographie: Chronologie einer Analysentechnik, GIT-Verlag, Darmstadt, 1995, p. 337.
[11] M. Hedenmo, B.M. Eriksson, J. Chromatogr. A 661 (1994) 153.
[12] F. Hasler, D. Bourquin, R. Brenneisen, T. Bär, F.X. Vollenweider, Helv. Pharm. Acta 72 (1997) 175.
[13] A.D. Batcho, W. Leimgruber, Org. Synth. 63 (1985) 214.

[14] R. Brenneisen, S. Borner, Arch. Pharm. 321 (1988) 487.
[15] W. Funk, V. Damann and G. Oehlmann, Statistische Methoden in der Wasseranalytik, VCH Verlagsgesellschaft, Weinheim, 1985, p. 32.
[16] D. Chollet, M. Salomon, J. Chromatogr. 593 (1992) 73.
[17] W. Funk, V. Damann and G. Oehlmann, Statistische Methoden in der Wasseranalytik, VCH Verlagsgesellschaft, Weinheim, 1985, p. 61.
[18] L. Sachs, Applied Statistics, Springer Verlag, New York, 1982, p. 312.
[19] H.P. Koch and W.A. Ritschel, Synopsis der Biopharmazie und Pharmakokinetik, Ecomed Verlagsgesellschaft, Landsberg, 1986, p. 308.