Determination of methadone and its metabolites EDDP and EMDP in human hair by headspace solid-phase microextraction and gas chromatography–mass spectrometry

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Abstract

A simple method for analysis of methadone and its two main metabolites EDDP and EMDP in hair was developed using automatic headspace solid-phase microextraction (HS-SPME) at a multipurpose sampler and gas chromatography – mass spectrometry with electron impact ionization and selected ion monitoring (GC–MS-SIM). The washed hair pieces were digested in the closed headspace vial in 1 ml 1 M NaOH containing 0.5 g NaCl and each 10 ng of the internal standards D_9-methadone and D_3-EDDP at 110°C for 20 min. Then the HS-SPME was performed with a 65 µm polydimethylsiloxan/divinylbenzene fiber at the same temperature in the same vial for another 20 min followed by the desorption in the GC injection port. The calibration curves were linear between 0.1 and 3 ng/mg (methadone and EMDP) and 10 ng/mg (EDDP) respectively, at higher concentrations a negative deviation from linearity was found. The detection limits were 0.03 ng/mg (methadone) and 0.05 ng/mg (EDDP and EMDP), and the reproducibility was 9.2% for methadone and 11.2% for EDDP ($n=12$). The method was applied to hair samples of 26 drug fatalities. 19 cases were positive with 0.36–11.8 ng/mg methadone and 0.19 –10.8 ng/mg EDDP. EMDP was found only in two cases with 0.18 and 0.84 ng/mg. The methadone concentration range was in agreement with previous data, but the EDDP/methadone concentration ratios (0.19–0.67) were definitely higher than those determined by other methods. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Methadone; EDDP; EMDP

1. Introduction

Methadone is an opioid analgesic. In many countries it is used for substitution therapy of heroin addiction. As a result of the very liberal prescription practice it gets into the drug scene and is abused also by other persons leading to severe intoxications and methadone fatalities [1–3]. Besides the regular control of urine, blood or saliva samples [4–8] hair analysis proved to be a suitable way for a retrospective long-term control of the intake or abuse also for this drug [9–14]. The main metabolites 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenylpyrroline (EMDP) are formed by subsequent enzymatic removal of both *N*-methyl groups and cyclization (Fig. 1).

Different methods were described in literature for analysis of methadone and EDDP from hair. Marsh et al. applied a radioimmunoassay to extracts of the hair [9]. Gas chromatography–mass spectrometry

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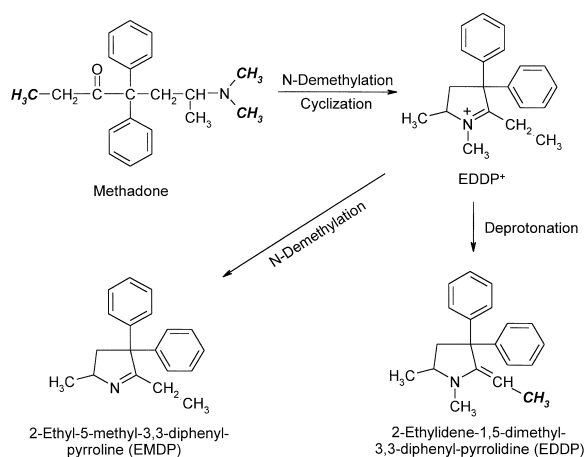*E-mail address:* fritz.pragst@charite.de (F. Pragst).

Fig. 1. Structure of methadone and its main metabolites EDDP and EMDP. The italic printed methyl groups are deuterated in the internal standards D_9-methadone and D_3-EDDP.

(GC–MS) was used by Moeller et al. after extraction of the hair samples with aqueous buffer (pH 7.6) and subsequent clean-up by solid-phase extraction (SPE) [10] and by Goldberger et al. after methanol extraction and clean-up by SPE [11]. Wilkins et al. analyzed methadone and the two metabolites by digesting the hair samples with 1 *M* NaOH, subsequent liquid–liquid extraction with butylchloride–acetonitrile and GC–MS with positive chemical ionization [12]. Liquid chromatography–ion spray-mass spectrometry (LC–MS) with a chiral column after enzymatic hydrolysis of the pulverized hair samples and SPE was applied by Kintz et al. for the enantioselective determination of *R*- and *S*-methadone [13]. Lucas et al. separated methadone and EDDP by solid-phase microextraction (SPME) after 12 h treatment of the hair with pronase E and dilution with borax buffer before measurement by GC–MS [14]. In this case the extraction by the fiber occurred directly from the liquid phase.

SPME was developed by Pawliszyn [15] and proved to be a very convenient and solvent saving way of sample preparation for GC–MS in numerous applications. The principle and the practical details of this extraction by polymer coated fibers were described several times [16–18]. Besides the direct extraction of the analytes from the aqueous solution, the headspace mode of SPME (HS-SPME) appeared to be particularly advantageous for volatile and

''semi-volatile'' substances because of the very low chromatographic underground. Surprisingly, also compounds with a boiling temperature up to 300°C could be extracted via headspace below 100°C with absolute yields between 0.04 and 8.5% within 10 to 60 min. Examples of such applications of HS-SPME to blood or urine samples are the determination of amphetamines [19–21], phencyclidine [22], tri- and tetracyclic antidepressants [23,24], local anesthetics [25], phenothiazines [26] or diphenylmethane antihistaminics [27]. HS-SPME was first applied to hair analysis by Koide et al. [28], who determined amphetamine and metamphetamine from only 1 mg hair with detection limits of 0.1 and 0.4 ng/mg respectively. Lidocaine as a frequent adulterant in illicit cocaine and heroin preparations were analyzed by HS-SPME and GC–MS in hair samples of 32 drug fatalities by Sporkert et al. [29]. It was shown by the same authors at the example of more than 20 drugs that for lipophilic basic compounds, which are stable to hydrolysis, the combination of alkaline hair digestion and HS-SPME within one step can be superior to the other methods with respect to manual, time and material expenses and provides the same detection limits and reproducibility [30].

In this paper a simple and fast method for analysis of methadone and its main metabolite EDDP from hair by HS-SPME and GC–MS and its application to hair samples of 26 drug fatalities are described. In cases with very high methadone consumption also the second metabolite EMDP can be measured by this method. It is shown that alkaline hair digestion, HS-SPME and GC–MS measurement can automatically be performed using the multipurpose sampler MPS 2 in combination with the GC–MS device.

2. Experimental

2.1. Hair samples

The scalp hair samples analyzed in this investigation were collected by cutting directly above the skin from 26 fatalities who died in 1997 and 1998 after drug overdose and were post mortem examined at the Institute of Legal Medicine at the Humboldt-University Berlin. Only in 13 of these cases the methadone consumption was known from the case histories or from the blood and urine analyses.

For the development of the method a pool of drug-free hair from colleagues and friends was used, who did not intake any drugs.

In order to remove external contamination the samples were washed each 5 min with water and with acetone in an ultrasonic bath and dried. Then they were cut to pieces of 1–2 mm length.

This investigation was allowed by the ethics commission of the university hospital Charité of the Humboldt-University.

2.2. Reagents

Standards in ampoules (1 mg/ml or 0.1 mg/ml in methanol or acetonitrile) of methadone, D_9-methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine (EDDP), D_3-EDDP and 2-ethyl-5-methyl-3,3-diphenylpyrroline (EMDP) were purchased from Promochem (Wesel, Germany). These standard solutions were diluted to the concentrations 10 µg/ml, 1 µg/ml and 0.1 µg/ml with water for calibration or for addition of the internal standards to the samples.

All other reagents were obtained in analytical grade purity from Merck (Darmstadt, Germany).

2.3. Instruments

A gas chromatograph 6890 with a mass selective detector 5973 (Hewlett-Packard GmbH, Waldbronn, Germany) was used for the GC–MS measurements. This was combined with a multipurpose sampler MPS 2 (Gerstel, Mühlheim/Ruhr, Germany), with which all steps of the HS-SPME experiments (preheating of the sample and headspace adsorption in the heating station with or without sample agitation, desorption in the GC injection port) could be programmed and automatically carried out. An ultrasonic bath Sonorex (Bandelin electronic, Berlin, Germany) was used for cleaning the hair samples before analysis.

2.4. Hair hydrolysis and HS-SPME

To 10 mg of the washed and dried hair pieces in 10 ml-headspace vials 0.5 g NaCl, 1 ml 1 *M* NaOH and each 10 ng of D_9-methadone and D_3-EDDP (10 µl of the 1 µg/ml solution) were added. Then the vials were tightly closed with silicon/PTFE septa and steel caps and placed into the vial rack of the multi purpose sampler.

The SPME fibers fitting to the MPS 2 were obtained from Supelco (Deisenhofen, Germany). For the optimization of the SPME method a 65 µm polydimethylsiloxan/divinylbenzene fiber (PDMS/DVB) and a 85 µm polyacrylate fiber (PA) were used. The routine analyses were performed with the 65 µm PDMS/DVB fiber.

After optimization the following conditions were used for hair hydrolysis and HS-SPME: Preheating 20 min at 110°C and 300 rpm agitation, headspace adsorption 20 min at 110°C and 150 rpm agitation, desorption 5 min at 260°C (PDMS/DVB fiber) or 290°C (PA fiber). The agitation mode was 60 s right, 15 s interval, 60 s left, 15 s interval etc.

2.5. Gas chromatography–mass spectrometry

An HP-5-MS capillary column (30 m×0.25 mm× 0.25 µm, Hewlett-Packard, Waldbronn, Germany) with Helium 5.0 as carrier gas (1.0 ml/min) was used for the gas chromatographic separation. The injection mode was splitless for 3 min. The following temperature program was applied: 2 min at 100°C, then 20°/min up to 300°, then 2 min at 300°C. The temperatures of the injector, the interface, the ion source and the quadrupole were 260°C (290°C), 280°C, 230°C and 106°C respectively.

For the detection in the selected ion mode (SIM, three time windows) the following m/z values were chosen: methadone 72 and 294, D_9-methadone 78 and 303, EDDP 262, 276 and 277, D_3-EDDP 265, 279 and 280 and EMDP 115, 130 and 208. The retention times were 9.49 min for EMDP, 9.96 min for EDDP, 9.94 min for D_3-EDDP, 10.45 min for methadone and 10.42 min for D_9-methadone. As a typical example in Fig. 2 the SIM-chromatograms for the hair sample 164/98 is shown. EMDP was not found in this sample.

3. Results and Discussion

3.1. Optimization of the HS-SPME/GC–MS method

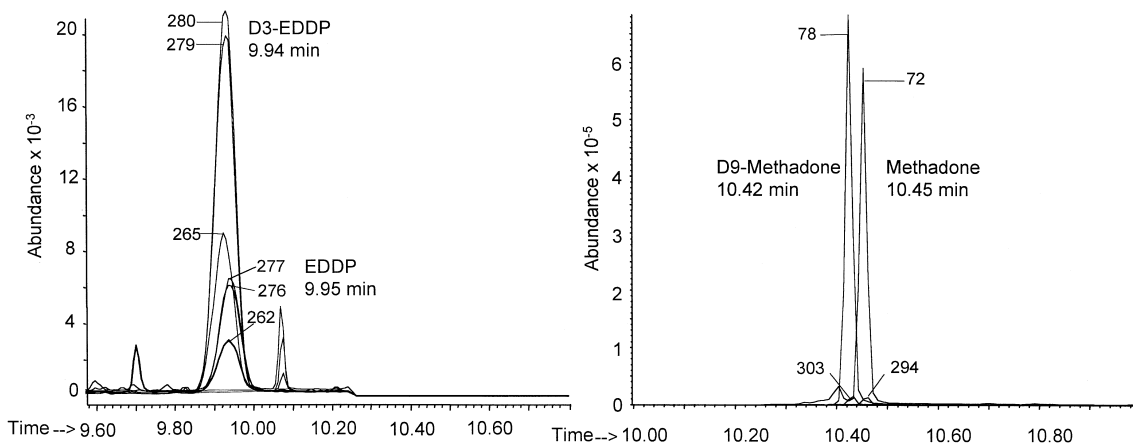Since methadone as well as EDDP and EMDP are stable in alkaline medium a combination of hair

Fig. 2. GC–MS-SIM chromatogram of the hair sample 164/98 (10 mg) with 10 ng D_9-methadone and 10 ng D_3-EDDP as internal standards. Concentrations: methadone 0.74 ng/mg, EDDP 0.29 ng/mg.

digestion in aqueous NaOH and HS-SPME should be possible if the volatility of both compounds under the analytical conditions is high enough. For optimization of the method the effect of the essential analytical parameters on the HS-SPME extraction yield was investigated for methadone and EDDP. At first the influence of the SPME fiber, of the digestion medium, of the adsorption time and of the adsorption temperature were studied in absence of hair. After that the effect of the hair matrix was investigated at spiked hair samples and the calibration and validation of the method were carried out.

3.1.1. SPME fiber

According to the recommendation of the manufacturer the 65 μm polydimethylsiloxan/divinylbenzene fiber (PDMS/DVB) and the 85 μm polyacrylate fiber (PA) should be suitable for this problem. The comparison of the peak areas as a measure of the extraction yields is shown in Fig. 3. It is seen that methadone is about 20% more effectively extracted by the PA fiber, whereas the EDDP yield is about 25% higher with the PDMS/DVB fiber. From literature data a lower concentration of EDDP was expected [10–14]. Therefore the PDMS/DVB fiber was chosen for the further investigations.

3.1.2. Composition of the solution for hair digestion

Besides the liberation of the analytes from the hair matrix the composition of the reagents has also an

effect on the HS-SPME extraction yield, particularly by a salt-out effect. Therefore different concentrations of NaOH and the addition of excessive Na_2SO_4 or NaCl were tested. The results are shown in Fig. 4. Altogether there are no very strong differences. Increase of the NaOH concentration from 1 to 7.5 M leads to a decrease particularly of the methadone yield. Addition of 0.5 g NaCl leads to a somewhat higher extraction yield than addition of 0.5 g Na_2SO_4. Therefore in the further experiments 1 ml 1 M NaOH+0.5 g NaCl were used.

3.1.3. Adsorption time and adsorption temperature

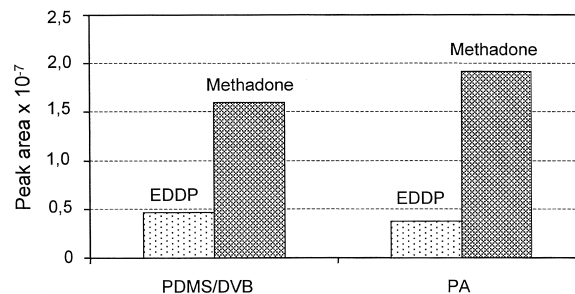The effect of the adsorption temperature between 60 and 120°C and of the adsorption time between 5

Fig. 3. Comparison of the 65 μm polydimethylsiloxan/divinylbenzene fiber (PDMS/DVB) and the 85 μm polyacrylate fiber (PA) for HS-SPME analysis of methadone and EDDP (n=2). Solution: 100 ng methadone and 100 ng EDDP in 1 ml 1 M NaOH+0.5 g Na_2SO_4, preheating: 5 min at 90°C, adsorption: 20 min at 90°C.
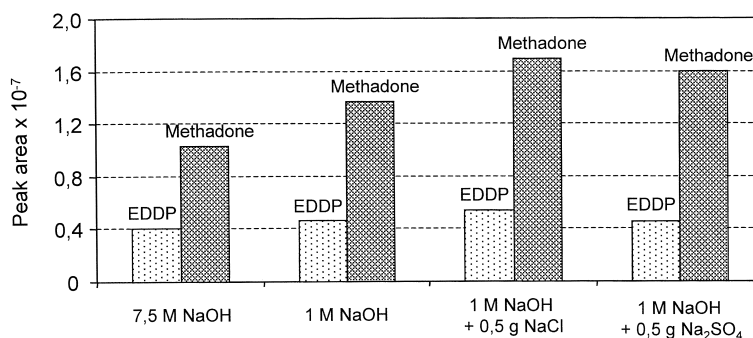
Fig. 4. Effect of the composition of the solution for hair digestion on the HS-SPME efficiency ($n=2$). Fiber: PDMS/DVB, solution: 100 ng methadone and 100 ng EDDP in 1 ml 1 M NaOH+0.5 g NaCl, preheating: 5 min at 90°C, adsorption: 20 min at 90°C.

and 50 min are shown in Fig. 5. Up to 100°C there was a steady increase of the extraction yield for both compounds. Between 100 and 110°C an additional strong growth of the yield was found for methadone and to a lower extent also for EDDP. Further increase of the temperature was without effect. Therefore, 110°C were chosen for the routine measurements.

With rising adsorption time up to 30 min the extraction yield increases. After that a slow decrease was found. As a reason a slow decomposition of the substances is possible. As a reasonable compromise the further investigations were carried out with 20 min adsorption time.

3.1.4. Sample amount

It was found in an earlier investigation that the extraction yield strongly depends on the amount of hair matrix dissolved in the alkaline medium [30]. In order to examine this effect for methadone and EDDP 100 ng of both substances were investigated in presence of 0, 3, 10, 30 and 100 mg of drug-free hair. For dissolution of the hair in these experiments the preheating time was extended to 20 min. It can be seen from Fig. 6 that the methadone yield strongly decreases with increasing amount of hair. In presence of 100 mg hair it is only about 10% of the value in absence of hair. On the other hand, the yield of EDDP is at first increased by the hair matrix and only above 10 mg hair decreases.

This strong matrix effect can be explained in the following way: the lipophilic analytes have a very high concentration at the surface of the aqueous solution, which is increased by the salt-out effect. This high surface concentration is particularly important for the headspace extraction of compounds
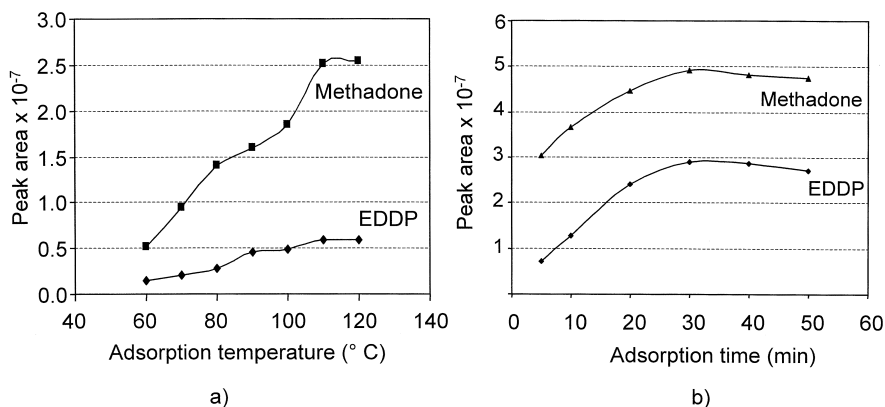

Fig. 5. (a) Effect of the adsorption temperature (time 20 min). (b) Effect of the adsorption time (temperature 110°C) on the HS-SPME efficiency. Fiber: PDMS/DVB, solution: 100 ng methadone and 100 ng EDDP in 1 ml 1 M NaOH+0.5 g NaCl, preheating: 5 min at the adsorption temperature, $n=2$.
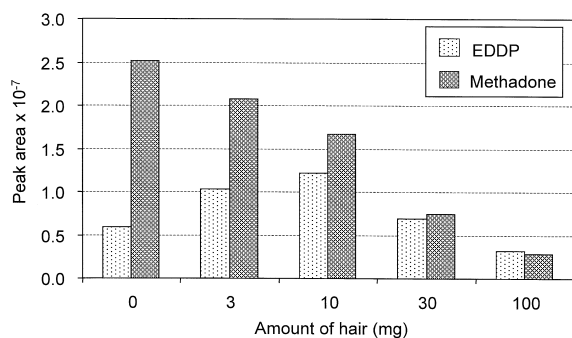
Fig. 6. Effect of the amount of hair on the HS-SPME extraction yield of methadone and EDDP. Fiber: PDMS/DVB, solution: 100 ng methadone and 100 ng EDDP in 1 ml 1 M NaOH+0.5 g NaCl, addition of 0, 3, 10, 30 and 100 mg drug-free hair, preheating: 20 min at 110°C, adsorption 20 min at 110°C, n=2.

with a very low volatility. In presence of hair the surface concentration of the analytes may be diminished by displacement by other lipophilic dissolution products of the matrix or by increase of the solubility caused by surface active hair hydrolysis products. This is the case for methadone and at higher hair amounts also for EDDP. On the other hand, at low sample amounts the surface concentration of EDDP is obviously increased by hair hydrolysis products.

From the results it follows that an optimal sample amount is 10 mg and all further measurements were performed with this amount. An increase of the sensitivity by investigating higher sample amounts is only partially possible because of the decreasing extraction yields. Furthermore, with 100 mg hair the solution was very viscous. That may lead to a longer equilibration time.

By use of deuterated internal standards the matrix effect on the extraction yield should be irrelevant for the quantitative result. However, if other internal standards with a possible different matrix effect are used, always the same sample amount must be investigated for calibration as well as for sample analysis.

3.1.5. Calibration

For calibration 10 mg drug-free hair were spiked with 11 different concentrations between 0.03 and 30 ng/mg of each methadone and EDDP and analyzed in presence of 10 ng D_9-methadone and D_3-EDDP as

the internal standards. For EMDP seven different concentrations between 0.03 and 3 ng/mg were measured with D_3-EDDP as the internal standard. The calibration curves are shown in Fig. 7. At concentrations up to 3 ng/mg for methadone and for EMDP and up to 10 ng/mg for EDDP linearity was found (r^2=0.9999, 0.9990 and 0.9974 respectively). Above these concentrations a deviation from the linearity is observed, which is stronger for methadone than for EDDP. The reason could not be established. In the case of methadone concentrations above 10 ng/mg, the measurement should be repeated with a smaller sample amount in order to get into the linear region.

3.1.6. Limit of detection (LOD) and limit of quantification (LOQ)

According to the German Industrial Norm (DIN 32645) the LOD and the LOQ were estimated as the threefold and the 11-fold standard deviation of the base line noise respectively. LOD was 0.03 ng/mg for methadone and 0.05 ng/mg for EDDP and EMDP, and LOQ was 0.10 ng/mg for methadone and 0.16 ng/mg for EDDP and EMDP. These LOD and LOQ values were confirmed by measurement of spiked hair samples in the concentration range between 0.03 and 0.3 ng/mg.

3.1.7. Reproducibility

By automatic performance of all HS-SPME steps with the multipurpose sampler temperatures and times could be much better controlled and held constant than by manual performance. Therefore also the absolute peak areas were well reproducible. For 16 measurements with spiked hair samples (10 mg) the intra-day coefficient of variation was 4.88% for methadone and 7.21% for EDDP. EMDP was not involved in this series.

For determination of the reproducibility of the whole analytical procedure the hair sample of the case 316/97 was measured 12 times within 3 days. The following results were obtained:

Mean concentration: methadone 2.66 ng/mg
EDDP 0.67 ng/mg
Standard deviation: methadone 0.25 ng/mg (9.2%)
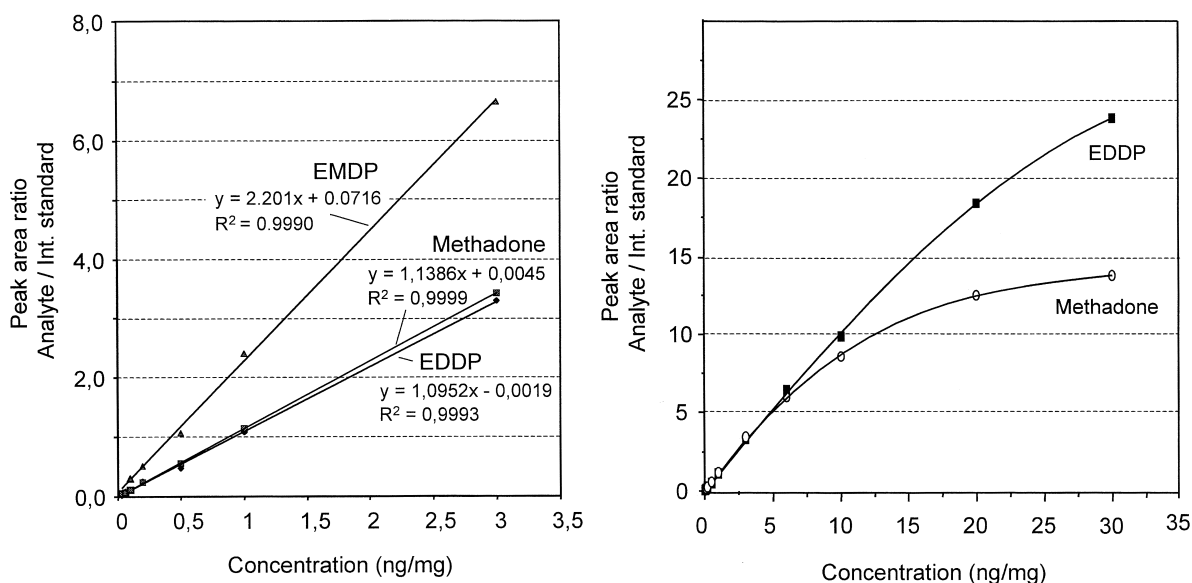EDDP 0.08 ng/mg (11.8%)

Fig. 7. Calibration curves of methadone, EDDP and EMDP. Fiber: PDMS/DVB, solution: 10 mg drug-free hair, 10 ng D$_9$-methadone and 10 ng D$_3$-EDDP in 1 ml 1 M NaOH+0.5 g NaCl, preheating: 20 min at 110°C, adsorption 20 min at 110°C, n=2. Concentrations: 0.03, 0.06, 0.1, 0.2, 0.5, 1.0, 3.0, 6.0. 10, 20 and 30 ng/mg, in the case of EMDP only up to 3.0 ng/mg.

3.1.8. Absolute extraction yields

The absolute HS-SPME extraction yield was calculated from the peak area obtained in the HS-SPME experiment in comparison to the peak area measured after direct injection of the same substance amount. Using the optimized procedure with 10 mg drug-free hair spiked with 10 ng of each methadone, EDDP and EMDP the absolute extraction yields in
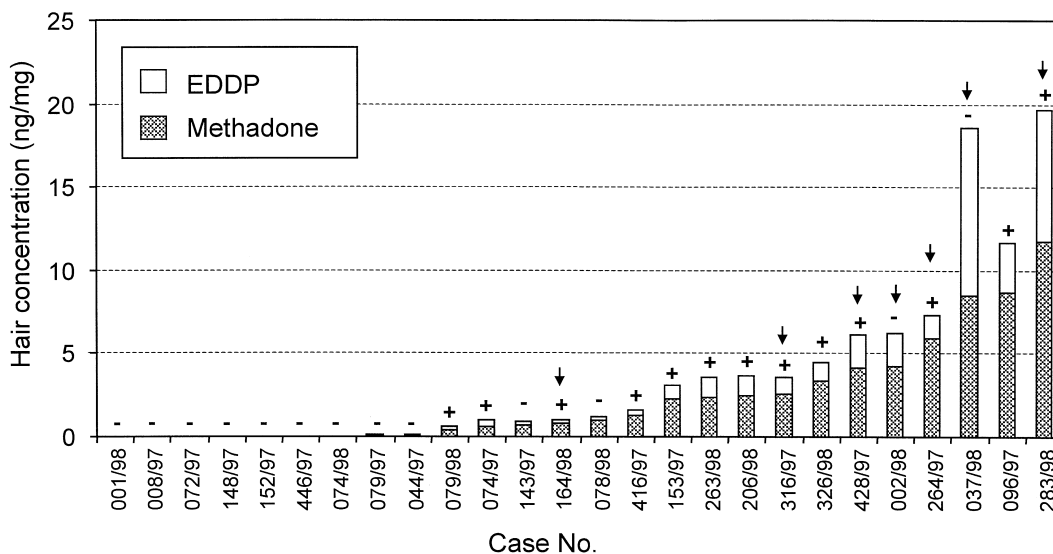

Fig. 8. Concentrations of methadone and EDDP in hair of 26 drug fatalities. In the cases marked by " + " the consumption of methadone was known from the case history or from the investigation of the blood and urine samples. The cases marked by "↓" were also analyzed for EMDP.

four measurements were 10.5–11.2% for methadone, 11.0–14.5% for EDDP and 15.9–17.4% for EMDP. Yields in this range were also found for other compounds [30].

3.2. Application to hair samples of drug fatalities

The method was applied to hair samples of 26 drug fatalities of the years 1997 and 1998. Only in 13 of these cases the consumption of methadone was known either from the case histories or from the results of the blood and urine analysis. Always the 3 cm long proximal hair segment was investigated. The concentrations are shown in Fig. 8. Methadone was detected in 19 cases with 2 cases below the LOQ and EDDP was found in 17 cases. In all 13 cases with a known methadone consumption also, a

positive hair analysis result was obtained. In the other 6 positive cases the uptake of this drug was only proved by hair analysis.

The analysis for EMDP was carried out only with seven of the samples with a positive methadone result. These cases are marked by a "↓" in Fig. 8. EMDP was detected only in the cases 037/98 and 383/98 with concentrations of 0.18 and 0.84 ng/mg respectively. In Fig. 9 the SIM chromatograms obtained for case 037/98 are compared with the extracted ion chromatograms of the standard. EMDP is identified by agreement of the retention time as well as of the peak area ratio for the three m/z values chosen.

The concentrations were between 0.36 and 11.8 ng/mg for methadone and between 0.19 and 10.1 ng/mg for EDDP. For comparison in four cases the
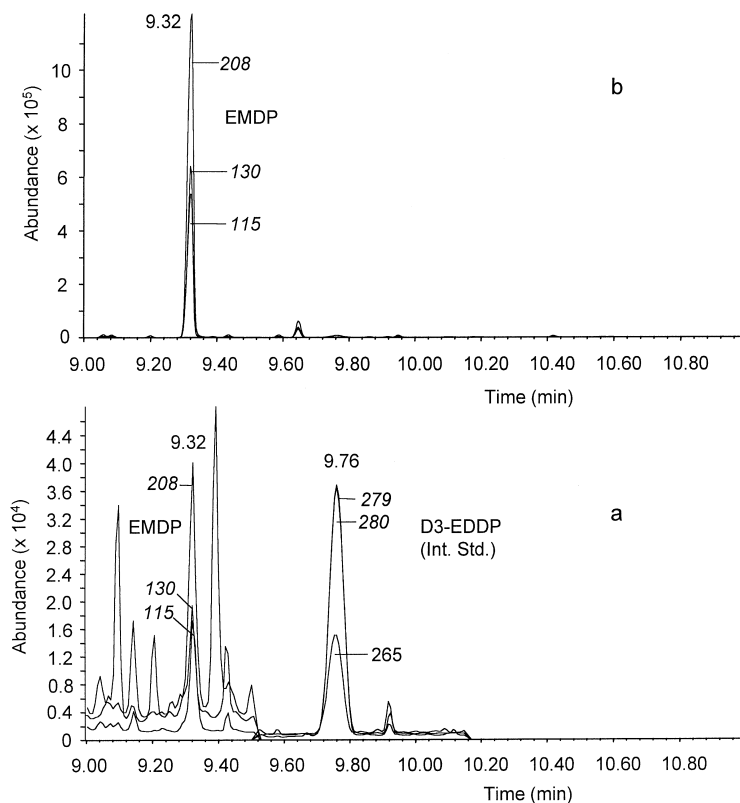

Fig. 9. (a) Detection of EMDP (0.18 ng/mg) in the hair sample 037/98 in the SIM chromatograms with D_3-EDDP (1 ng/mg) as internal standard. (b) Extracted ion chromatograms from the total ion measurement of EMDP (100 ng in absence of hair). The difference in retention times as compared to the experimental part and Fig. 2 is caused by a shortening of the capillary column.

methadone concentrations were also determined with a second method [29] by extraction of the ground hair with buffer pH 6, subsequent solid-phase column extraction (SPE) and GC–MS of the extract. The method did not involve EDDP and EMDP. The results are shown in Table 1. Regarding the numerous sources of error in quantitative hair analysis the results are in sufficient agreement.

The range of the methadone concentrations is in agreement with the data described by other authors, but the EDDP concentrations are clearly higher than those described in literature [10–14]. With the exception of two extreme cases (037/98 and 283/98) the concentration ratio of the metabolite EDDP and the parent drug methadone was between 0.19 and 0.67 (medium 0.39, literature data 0.05 to 0.23 [10,12,14]). EMDP was detected for the first time in human hair. Wilkins et al. obtained a negative result for two human subjects with 10.1 and 21.0 ng/mg methadone in hair and with an LOQ of 1 ng/mg [12]. In the hair of a rat fed with methadone, EMDP was positive with a concentration <0.5 ng/mg. Also in forensic blood samples it could not be detected [6]. Therefore the small concentrations in the two cases and the negative results of the other samples analyzed for EMDP are not surprising.

Altogether the HS-SPME method proved to be very reliable and sufficiently sensitive for the routine analysis of methadone and its metabolite EDDP in hair. The second metabolite EMDP can be detected in cases with very high methadone concentration. By use of the multipurpose sampler MPS 2 the manual work was minimized and the reproducibility was improved. Furthermore, in contrast to other methods no organic solvents were necessary.

Table 1
Comparison of the methadone concentrations in hair of four drug fatalities determined by GC–MS after HS-SPME or after buffer extraction and subsequent SPE

Case No.	Methadone concentration (ng/mg)	
	HS-SPME/GC–MS	SPE/GC–MS
264–97	5.94	7.98
428–97	4.10	4.76
002–98	4.21	3.55
283–98	11.75	10.70

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