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Determination of fentanyl in human plasma by head-space solid-phase microextraction and gas chromatography–mass spectrometry

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Abstract

A head-space solid-phase microextraction (HS-SPME) method coupled to GC–MS was developed to extract fentanyl from human plasma. The protein binding was reduced by acidification and, eventually, the sample was deproteinized with trichloroacetic acid. The parameters influencing adsorption (extraction time, temperature, pH and salt addition) and desorption (desorption time and temperature) of the analyte on the fibre were investigated and validated for method development. The developed method proved to be rapid, simple, easy and inexpensive and offers high sensitivity and reproducibility. Linear range was obtained from 0.1 ng/ml to 2 μg/ml. The limit of detection was 0.03 ng/ml while an inter-day precision of less than 5% ($n = 15$) could be achieved. The method has been applied for the determination of fentanyl in plasma samples after application of 50 μg/h Duragesic fentanyl patch.

Keywords: Sol–gel technology; Solid-phase microextraction; Gas chromatography–mass spectrometry; Fentanyl

1. Introduction

Fentanyl, *N*-(1-phenethyl-4-piperidyl) propionanilide (Fig. 1), is a potent synthetic opiate commonly used for surgical analgesia and sedation. It is approximately 50–100 times more potent than morphine and has a rapid onset (1–2 min), but short duration of action (30–60 min). Fentanyl has minor cardiovascular effects but can induce respiratory depression, hypotension, and coma. Because of its potency and quick onset, even a very small dose of fentanyl can lead to sudden death; the minimal lethal dose for fentanyl is estimated to be 2 mg [1–5].

Comprehensive pharmacokinetic studies of fentanyl have proven difficult as the blood concentration of fentanyl from single or infrequent doses falls rapidly below the limit of detection (LOD) of most assay procedures. In order to study the pharmacokinetic of fentanyl, its detection at lower level of analgesic doses is rather important.

A number of methods have been developed to measure fentanyl concentration in biological fluids with different levels of sensitivity and usefulness in the pharmacokinetics of fentanyl. Enzyme-linked immunosorbent assay (ELISA), radiochemical and radioimmunoassay [4,6–8] methods have been utilized for detection of fentanyl with limit of detection ranging from 1.05 to 20.0 pg/ml, but these methods have low precision or suffer from lack of selectivity, particularly at clinically relevant levels of fentanyl. High-performance liquid chromatography (HPLC) with UV detection [9–12], liquid chromatography with tandem mass spectrometry (LC–MS/MS) [12–16], gas chromatography (GC) with nitrogen-phosphorus (GC-NPD) [3,17,18] and mass spectrometry (GC–MS) [4,5,19] have been also developed. While HPLC-UV and in some cases GC-NPD have shown poor sensitivity, the others have sufficient sensitivity for pharmacokinetic studies. The limit of detection values in these methodologies varied from 2.5 to 400 pg/ml. Usually, liquid–liquid extraction (LLE) and solid-phase extraction (SPE) [13,14] are the most common techniques for isolation and/or enrichment of fentanyl prior to chromatographic analysis. These methods have many disadvantages, as they are tedious, labor-intensive and time-consuming. LLE produces an emulsion and requires the use of

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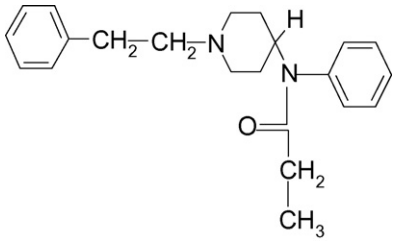

Fig. 1. Chemical structure of fentanyl.

large amount of highly-purified solvents, which are often hazardous and result in the production of toxic laboratory waste. Prior to the chromatographic analysis, when LLE and SPE are employed, there is a need for solvent evaporation, in order to preconcentrate the samples. Although SPE is less time-consuming than LLE, it still requires an appreciable amount of toxic solvent for analyte desorption.

SPME, nowadays, is a well-known sampling and sample preparation technique. This technique, initially introduced for the analysis of volatile compounds, has gained an increasingly important role for isolation of vast varieties of compounds from aquatic media [20–23]. This method effectively overcomes the difficulties of conventional extraction methods by eliminating the use of organic solvents and allowing sample extraction and preconcentration to be performed in a single run. The technology is more rapid and simple than the conventional methods. It is also inexpensive, portable and sensitive.

In this paper, a HS-SPME using home-made sol–gel based fibers, coupled with GC–MS was developed for the determination of fentanyl in human plasma. The SPME parameters were optimized using drug-free plasma samples spiked with fentanyl. To demonstrate the validation of the proposed method, the detection limit, linearity and precision were investigated.

2. Experimental

2.1. Chemicals and materials

Fentanyl citrate (purity > 99%) was kindly obtained from Dr. Abidi Laboratories (Tehran, Iran). A stock solution (100 μg/ml) of fentanyl was prepared in methanol, and stored at −20 °C. Other standard solutions were prepared daily by diluting this solution with double distilled water. Methanol, hydrochloric acid, sodium chloride, potassium carbonate, perchloric acid and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Pepsin, trichloroacetic acid (TCA), and zinc sulfate were purchased from Fluka (Buchs, Switzerland).

Plasma standards and three quality control (QC) samples were prepared by adding 100 μl of aqueous working standards to 0.9 ml of drug-free plasma to yield final desired concentrations.

Blood samples were obtained from a healthy human subject before and 2–6, 8 h after application of 50 μg/h Duragesic fentantyl patch. These were placed in heparinized blood collecting tubes and centrifuged at 5000 rpm for 10 min. The plasma was stored at −20 °C until analysis. Real samples were analysed to determine fentanyl concentration–time profile.

2.2. Apparatus

SPME syringe was purchased from Azar Electrode Co. (Uromieh, Iran). SPME fibers were prepared based on sol–gel technology according to previous work [21]. All samples were extracted from 4 ml clear glass vials with silicon septa and open-top phenolic caps. Samples were heated in a homemade glass water bath connected to a refrigerated circulating water bath (Neslab) and stirred using a Gerdhardt Bonn hot plate magnetic stirrer (Germany). A Heraeus Sepatech Model Labofuge 1500 centrifuge (Osterode/Harz, West Germany) was used to separate the supernatant from protein precipitate in deproteinization of plasma samples.

All gas chromatographic separations were performed on an Hewlett Packard (HP) 6890 plus series gas chromatograph, equipped with an HP 5973 mass selective detector (MSD) (Hewlett Packard, Palo Alto, CA, USA), and split–splitless injector. Helium was used at a flow rate of 1 ml/min as the carrier gas. The injection port temperature was 270 °C. The electron impact (EI) ion source, quadrupole mass analyzer, and the interface temperature were maintained at 230, 150, and 280 °C, respectively. The MS was operated in the selected ion monitoring (SIM) mode using *m*/*z* 245, 189 and 146 with dwell time of 100 ms for each ion. After performing extraction, the fiber was inserted into the GC injection port. A desorption time of 5 min was used for the analytes to be desorbed from the fiber and transferred into the GC column for analysis. The thermal desorption step was carried out in the splitless mode, maintaining the column temperature at 100 °C for 5 min. Low column temperature ensured effective solute focusing at the column inlet. On completion of the thermal desorption step, the split vent was opened and kept in that position for the rest of the chromatographic run. All separations were performed using an HP-1 column (Hewlett-Packard, Avondale, PA) with a 0.25 μm film thickness, 30 m × 0.25 mm. After completion of the sample introduction step, the column temperature was programmed to 220 °C at 20 °C/min, then to 280 °C at 10 °C/min, and held 5 min. The total run time was 22 min.

2.3. Deproteinization of plasma and SPME procedures

Plasma sample (1 ml) was acidified with 50 μl hydrochloric acid (37%) to disturb the fentanyl protein binding. Then, 100 μl TCA (100%, w/v) was added to denature the proteins. These processes eventually led to the precipitation of proteins. Subsequently, the samples were centrifuged at 10,000 rpm for 5 min. An amount of 0.8 ml of the supernatant was transferred to the silanized [21] SPME vial and mixed with 0.5 g sodium chloride and 1.2 ml solution containing 0.5 g potassium carbonate. The vials were sealed with an open-top phenolic cap and silicon septum. Sample vials were heated at 80 °C in a glass water bath, connected to a circulating water bath, and were stirred at 100% efficiency of magnetic stirrer. The pre-conditioned SPME fiber was exposed to the head-space and care was taken to prevent any direct contact between the fiber and the liquid sample. After 30 min extraction time, desorption was performed in 5 min at

270 °C. Prior to extraction the fiber was, daily, inserted in the hot injection port for 30 min.

3. Result and discussion

3.1. Precipitation of protein

Considering the theory of SPME and simplified model on drug protein binding [24,25], the amount of analyte extracted, n_f, in a quaternary system containing fiber, head-space, plasma-water and protein, can be described as the following equation:

$$n_f = \frac{K_{fw} V_f n_0}{K_{fw} V_f + K_{hw} V_h + K_{pr} n_{pr}^0 + V_w} \quad (1)$$

where n_0 is the initial amount of analyte in the sample, n_{pr}^0 the amount of protein in the sample and V_w, V_f, V_h are the volumes of sample, fiber coating and head-space, respectively. The parameters K_{fw}, K_{hw}, K_{pr} are related to fiber/sample, head-space/sample and protein/analyte equilibrium constants, respectively. The main problem of analysis by SPME in matrices containing protein can be concluded from Eq. (1), i.e., a decrease of sensitivity. This problem is prominent where binding affinity of the target analyte to protein is high. If plasma proteins are removed from the sample matrix, Eq. (1) will be changed to general equation (2) in SPME:

$$n_f = \frac{K_{fw} V_f n_0}{K_{fw} V_f + K_{hw} V_h + V_w} \quad (2)$$

As 80% of fentanyl is bound to plasma proteins [3,26,27], the plasma proteins were removed from samples prior to extraction. Different methods for protein denaturation and precipitation have been reported in literatures [3,4,28–31]. Perchloric acid, sodium hydroxide, pepsin, trichloroacetic acid along with hydrochloric acid, and zinc sulfate were used and evaluated for precipitation and denaturation of proteins. Since trichloroacetic acid and hydrochloric acid showed higher extraction efficiency in comparison with other reagents, they were selected for plasma sample pretreatment. Hydrochloric acid disturbs the fentanyl protein binding and trichloroacetic acid denatures the proteins.

3.2. Optimization of the SPME method

The equilibrium constants values in Eq. (2) are affected by temperature, salt content, pH and organic modifiers. SPME method development often involves a number of stages. A univariate approach was employed to optimize influential parameters, including extraction mode, sample volume, extraction temperature and time, fiber coating, agitation method, pH, salt and desorption condition. Some of these parameters could be set based on prior knowledge whereas the other ones required to be determined experimentally [24,25].

Since isolating the fiber coating from direct contact with the sample matrix would prevent and/or minimize adverse effect due to highly alkaline sample conditions, head-space extraction was preferred.

Due to the nature of biological samples and considering the SPME theory, 2 ml of extraction sample in a 4-ml vial was considered. This provided enough space for the fiber to be positioned in the head-space.

The method optimization was performed using 1 ml plasma containing 1 μg fentanyl based on the method described in Section 2.3.

3.2.1. Selection of fiber coating

Among four SPME fibers prepared using sol–gel technology [21], it was expected that PEG and Ucon coated fibers should show higher extraction efficiency due to close polarity to fentanyl. As it is shown in Fig. 2, the PEG 200 coated fiber showed higher extraction efficiency. The sol–gel coated Ucon and PEGs exhibited higher recoveries than PDMS. This is in agreement with our expectation regarding the polarity of Ucon, PEGs coated fibers and fentanyl. PEG 4000 coated SPME fiber has slightly higher recovery than PDMS and lower recoveries than PEG 200 and Ucon coated SPME fibers. These phenomena are due to lower porosity of PEG 4000 coated fiber.

While PEG and Ucon coated fibers showed higher recovery than PDMS but they demonstrated insufficient stability during developing process. These coatings have etheric functional groups, which are susceptible to acid and base. As potassium carbonate has been used in extraction, which creates a pH of 11.5–12, these coated fibers had shown insufficient stability. It means that after at most 20 extractions they were exhausted. As PDMS coated fiber showed sufficient stability for more than 200 extractions and good recovery, PDMS coated fiber was used.

3.2.2. Extraction time and temperature

The extraction time profiles obtained at different temperatures (Fig. 3) revealed that at higher sampling temperatures the equilibrium is established faster. At 60–70 °C, analyte was not extracted at equilibrium levels. At temperatures between 80 and 95 °C, equilibrium extraction is achieved in 20–30 min. An extraction temperature of 85 °C in 30 min was, therefore, selected as the extraction temperature for future work, because it gave a higher recovery level and the equilibrium extraction time has been reduced to a practical level. These effects have been explained in other relevant works [25,30,32,33] and we have discussed it, more recently [21].

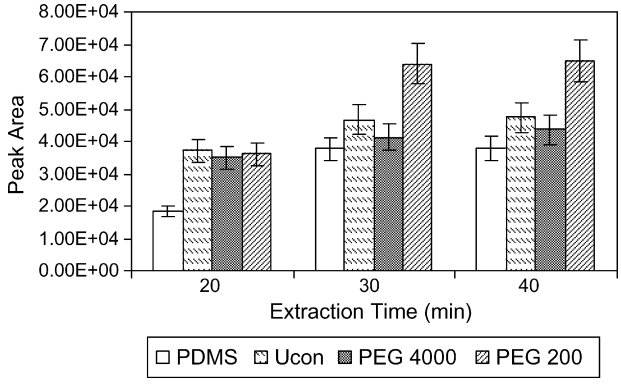

Fig. 2. Comparison of different fiber coatings for the analysis of fentanyl. Conditions were as described in Section 2.3.

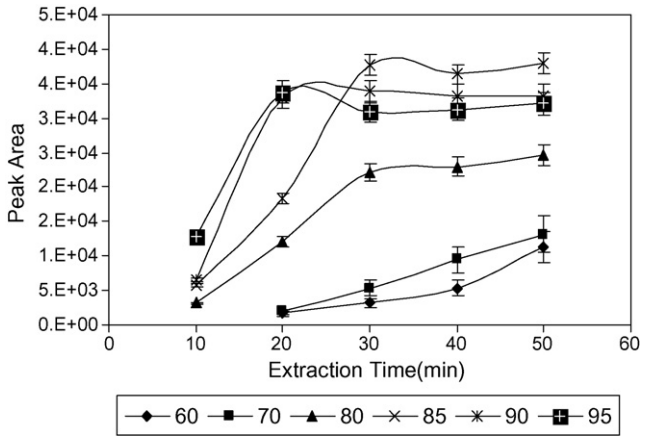

Fig. 3. Effect of temperature on absorption time profile, analyte recovery and equilibration time. Conditions described in Section 2.3 and PDMS fiber was used.

3.2.3. Effect of hydrochloric and trichloroacetic acids

Usually trichloroacetic acid is used for deproteinization. It was mentioned in some references that hydrochloric acid could disturb the drug–protein binding [34]. In order to study the effect of hydrochloric acid on extraction efficiency 0.0, 25, 50, 75 and 100 μl of this acid, was added prior to the addition of 100 μl of trichloroacetic acid solution. The maximum extraction efficiency was achieved when 50 μl of hydrochloric acid was used. In the absence of hydrochloric acid and even when, 25 μl was used, the extraction efficiency is low.

Effect of the amount of trichloroacetic acid was also investigated. The results showed that if 50 μl of trichloroacetic acid is added a maximum of extraction efficiency would be achieved. When less than 50 μl of trichloroacetic acid was used the deproteinization process could not be completed. After deproteinization and separation of protein precipitate, it is necessary to basify the supernatant which can result in increasing the salt concentration. When more than 50 μl of trichloroacetic acid and hydrochloric acid is used, the amount of salt will be increased and the extraction efficiency will be decreased.

3.2.4. Effect of pH and ionic strength

As Fig. 1 shows, fentanyl should be extracted under alkaline condition. Addition of potassium carbonate could neutralize the acids used in deproteinization process and adjust the pH value in the range of 11.5–12, which led to satisfactory extraction efficiency. This might be due to the pH and ionic strength effects caused by this divalent species. In order to check the whole alkaline pH ranges, sodium hydroxide, in the absence of potassium carbonate, was used. A decrease in extraction efficiency of analyte was observed for the alkaline pH ranges. Effect of potassium bicarbonate was also examined to obtain a rather lower pH, ranging from 8 to 9, but the results were poorer in comparison with the data obtained when potassium carbonate was used. Potassium carbonate was, therefore, selected for the pH adjustment. For SPME of biological fluids, appropriate buffer is necessary for the extraction and potassium carbonate is one of the best choices. Effect of potassium carbonate quantity on extraction efficiency was also tested using a range of 0.1–0.8 g of potassium carbonate per vial. Using 0.5 g of potassium carbonate per vial led to maximum efficiency. Excess amount of potassium carbonate will increase the ionic strength of solution causing a reduction in extraction efficiency.

In order to adjust the ionic strength, influence of sodium chloride addition on the efficiency of SPME was also investigated. Usually, the presence of salt increases the ionic strength of aqueous solution and would affect the solubility of organic solutes. In this experiment, sodium chloride concentrations of 0.3–0.7 g/vial were tested. An increase in extraction efficiency was observed by adding sodium chloride and the efficiency started to decrease when amounts higher than 0.5 g of NaCl/vial were employed. An amount of 0.5 g of NaCl/vial was therefore used as the optimum quantity. This confirms the results obtained in previous experiments. Fentanyl is not completely lipophilic in character and excess amount of sodium chloride will increase ionic strength and results a decrease in analyte volatility and extraction efficiency.

3.2.5. Desorption conditions

It is quite important to keep the time interval required for desorption as short as possible and carryover effects must be avoided. Thus, using the highest possible temperature without damaging the fiber coating and the smallest diameter of the injector insert should be applied. This is due to the fact that partition coefficient between fiber coating and head-space is decreased with increased temperature, and the linear flow rate is increased with smaller diameter of the insert. In all cases an insert with diameter of 0.75 mm was, therefore, used for SPME-GC–MS. The desorption temperature, was investigated in the range of 250–290 °C. The maximum extraction efficiency was obtained at temperatures higher than 270 °C. The desorption time was also optimized and after each desorption process, carry over effect was evaluated. Since desorption times below 4 min led to slight carryover, the desorption process was performed at 270 °C in 5 min.

3.3. Method validation

Preliminary works were carried out using the GC–MS in the full scan mode to check the retention time of analyte and any other possible co-extracted compounds. However, no interferences from co-extracted compounds were observed and eventually the GC–MS was operated in the SIM mode to enhance the sensitivity.

The optimized method was examined for the extraction and determination of fentanyl in plasma samples. The obtained chromatograms for blank, spiked and real samples from the same subject are shown in Fig. 4. The chromatogram of blank plasma sample reveals the absence of interfering peaks at the retention time of fentanyl.

Plasma samples were spiked with different concentrations of standard solutions, in a way that, final concentration of fentanyl was in the range of 0.01 ng/ml up to 3 μg/ml. After extracting fentanyl from plasma and performing GC–MS analysis, calibration curve for fentanyl was plotted. A linear range of 0.10 ng/ml

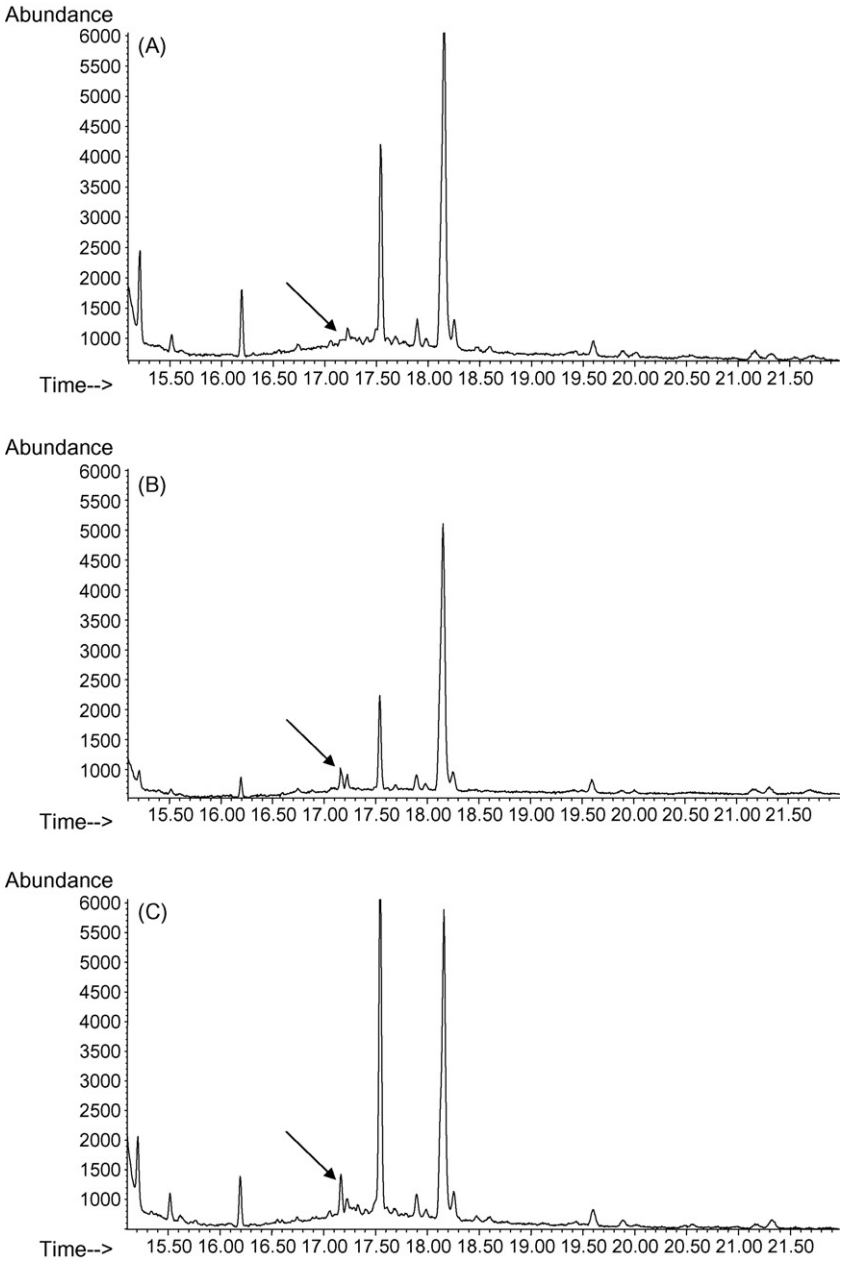

Fig. 4. Mass chromatograms obtained at *m/z* 245, 189 and 146 after SPME of (A) blank human plasma, (B) plasma spiked with 0.1 ng/ml fentanyl, and (C) authentic plasma sample collected at time point 8 h (0.87 ng/ml).

to 2 μg/ml with equation $y = 21.276x + 141.78$ and correlation coefficient (R^2) of 0.996 was obtained. Detection limit and limit of quantitation, based on a signal-to-noise ratio of S/N = 3 and S/N = 10, were 0.03 and 0.1 ng/ml, respectively. The accuracy was determined by comparing the means of measured concentrations with the nominal concentration for three levels of QC solutions. The precision was expressed as a mean percentage of the relative standard deviation (R.S.D.%). The results of the Intra-day and inter-day variation tests are presented in Table 1, which shows the R.S.D.% values are less than 5%.

Table 1
Intra-day and inter-day accuracy and precision of the SPME-GC–MS determination of fentanyl in plasma samples

Concentration (ng/ml)	Intra-day ($n = 5$)			Inter-day ($n = 15$)		
	Mean (ng/ml)	R.S.D.%	Accuracy (%)	Mean% (ng/ml)	R.S.D.%	Accuracy (%)
0.5	0.51	4.2	102	0.51	4.9	102
50	49.9	3.9	99.8	49.8	3.8	99.5
500	509	2.3	101.8	498	3.1	99.7

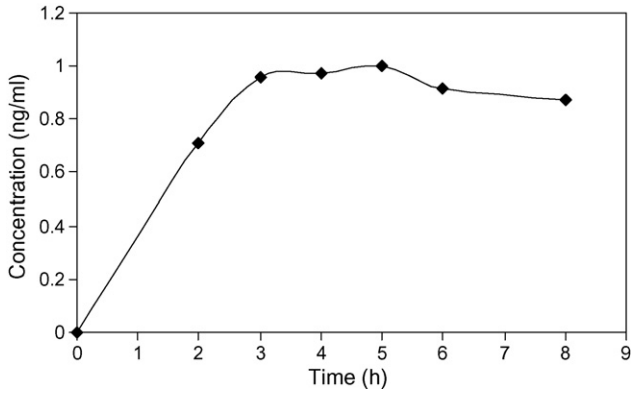

Fig. 5. Concentration of authentic samples from a volunteer after application of 50 μg/h Duragesic fentanyl patch.

3.4. Application

The described method was used to assess plasma concentration–time profile of fentanyl in human plasma after application of 50 μg/h Duragesic fentanyl patch. The plasma concentration curve obtained is shown in Fig. 5. The concentration could be determined for samples taken.

4. Conclusion

A head-space SPME-GC–MS method was developed for the determination of fentanyl in human plasma. It was shown that deproteinization is necessary for the determination of the total concentration of fentanyl in plasma. Various methods of deproteinization were applied and precipitation by hydrochloric and trichloroacetic acids was chosen for higher extraction efficiency. Influential parameters such as fiber coating type, extraction temperature and time, pH, ionic strength and desorption condition were investigated and optimized.

The proposed method provides a rather easy, simple, rapid and inexpensive SPME method for the determination of fentanyl with sufficient sensitivity and reproducibility. The use of automated SPME would definitely increase the speed of the method for higher throughput analysis.

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