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Highly sensitive gas chromatographic—mass spectrometric screening method for the determination of picogram levels of fentanyl, sufentanil and alfentanil and their major metabolites in urine of opioid exposed workers

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Abstract

Highly sensitive and specific analytical GC–MS procedures were developed and comprehensively validated for the determination of the opioid narcotics fentanyl, sufentanil and alfentanil and their major nor-metabolites in urine of potentially exposed opioid production workers. A simple, one step extraction protocol was developed using commercially available solid phase extraction (SPE) columns to recover all analytes from urine. The secondary amine functionalities of the nor-metabolites were derivatized to form stable, pentafluorobenzamide (PFBA)-derivatives with good chromatographic properties. Using the penta-deuterated analogues as internal standards, a limit-of-detection (LOD) of 2.5 pg fentanyl/ml, 2.5 pg sufentanil/ml and 7.5 pg alfentanil/ml urine was achieved. For the opioid metabolites the LODs were found to be <50 pg/ml urine. The developed analytical procedures show excellent intra-assay accuracy, particularly considering the ultra low levels of the analytes, with relative errors generally below 10%. Overall, an excellent reproducibility was observed with coefficients of variation below 10% at all spike levels for all opioid parent compounds and their metabolites, except for low norfentanyl concentrations. Upon storage at −30 °C urine samples were found to be stable for at least 2 months as no significant losses of either compound were observed. The developed analytical procedures have been successfully applied in a biological monitoring survey of fentanyl exposed production workers.

Keywords: Occupational exposure; Fentanyl; Sufentanil; Alfentanil

1. Introduction

Over the past decades, the development of the synthetic opioid narcotics fentanyl, sufentanil and alfentanil was an important step in anaesthetic management. Fentanyl, the first of the 4-anilinopiperidine series of opioid mu agonists, is chemically related to meperidine and has been reported to be 50–100 times more potent than morphine. Fentanyl was introduced into clinical practice in the early 1960s and its application as anaesthetic represented a major increase in the potency in comparison with the clinically important opiate agonists of the time [1,2]. In the early nineties, fentanyl became available in a transdermal therapeutic system that is designed to release the drug at a constant rate for up to 3 days. This non-invasive delivery of fentanyl has become significantly important in the treatment of general chronic and severe cancer related pain [3,4]. In recent years, a growing interest in alternative forms of drug administration has induced research on oral transmucosal and nasal fentanyl dosing systems [5,6]. Sufentanil and alfentanil, first synthesized in the mid seventies [7], are now also widely used to provide potent analgesia, as primary anaesthetic agents in very high doses during cardiac surgery, and in intensive care medicine [8]. Sufentanil is the most potent of the series and is about 5–10 times as potent as fentanyl, yet has a shorter duration of action. Alfentanil has the most rapid analgesic onset and exhibits about one third of the clinical potency of fentanyl [1,7]. In humans fentanyl, sufentanil and alfentanil are extensively metabolized and only a few per cents of the original doses are excreted unchanged in urine [9].

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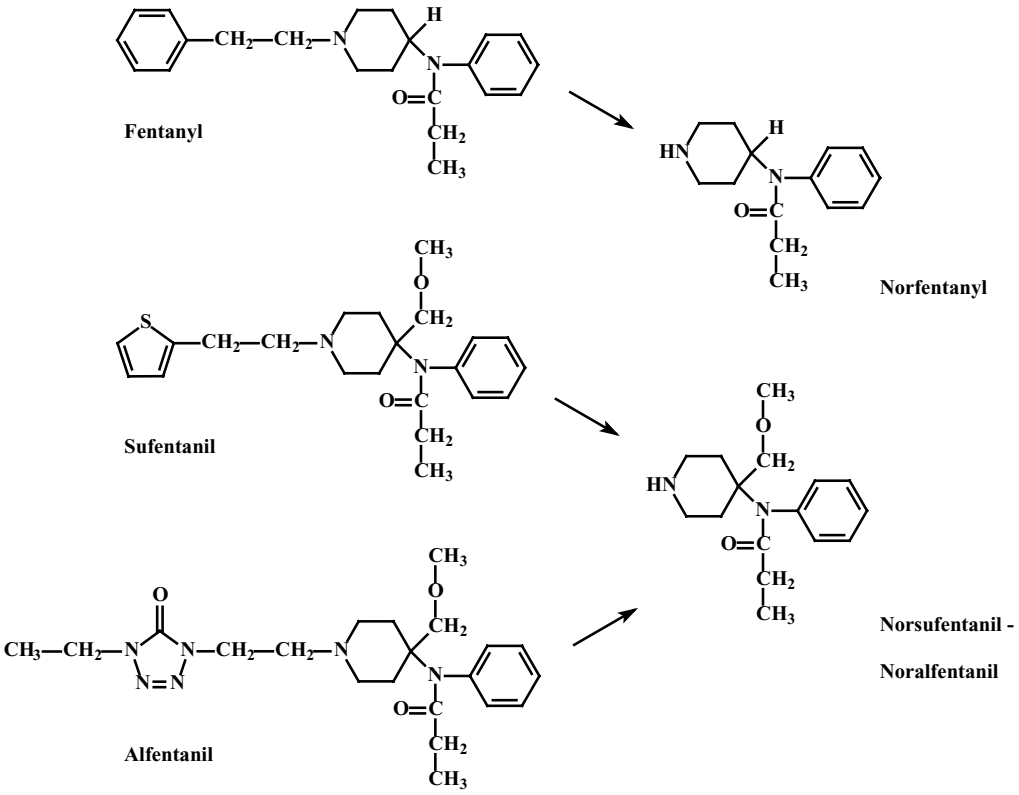

Fig. 1. Structure of the opioid narcotic analgesics and their respective nor-metabolites, formed through oxidative *N*-dealkylation.

The main metabolic pathway of the opioid analgesics is the oxidative *N*-dealkylation at the piperidine nitrogen, resulting in the formation of nor-metabolites (Fig. 1). In surgical patients receiving 0.5 mg fentanyl intravenously, 26–55% of the dose is excreted as norfentanyl in urine during the first 12 h [10]. Norsufentanil and noralfentanil, which are structurally identical, account for approximately 5 and 30% of the dose in the 0–24 h urine of patients after intravenous administration [9,11].

Prior to formulation in various dosage devices, fentanyl, sufentanil and alfentanil are synthesized as neat chemicals. As with other pharmaceutical ingredients that are specifically designed to modify biological function, production workers can be placed at risk of experiencing pharmacological effects if exposures are not adequately controlled. While these effects are considered desirable or controllable in patients treated for a particular medical condition, any clinically significant pharmacological effect occurring as a result of work exposure, is unacceptable [12]. Following exposure, primary adverse effects of the opioid analgesics may include dose-related sedation, associated with a risk of acute or delayed respiratory depression, bradycardia and hypotension [13]. Occupational hypersensitivity to opiates and cutaneous and respiratory responses from exposure to opiate compounds, like heroin and morphine, are reported in few publications [14–16]. To limit and control the potential exposure and the health risk associated of workers engaged in the synthesis and formulation of these potent narcotics, monitoring programs are required. In addition to industrial hygiene measurements for estimating external exposure to pharmaceuticals, biological monitoring aims at assessing the individual workers' uptake of the compound and the related risk. The advantages offered by biological monitoring in the occupational setting have been thoroughly reviewed by others and will not be merely repeated [17]. However, in the assessment of occupational exposure to opioid analgesics, one of the major advantages of biological monitoring is the fact that it takes into account absorption by other routes of exposure than the lungs. In view of the highly lipophilic nature of especially sufentanil and fentanyl and to a less extent of alfentanil [1,8] absorption through the skin could present an important concomitant route of exposure. Moreover, following ingestion, buccal liquefaction of these opioids could lead to a rapid absorption through the mucosa of the mouth, pharynx, and esophagus, [6,18], resulting in a potential contribution to the total exposure. Finally, fentanyl and alfentanil metabolism and clearance may be subject to inter-individual variability [19,20], and selecting the appropriate biomarker of exposure could potentially provide additional information on the individual susceptibility of exposed workers.

Because of their high potencies and the associated risks of intoxication, significant measures have been taken by the pharmaceutical industry to control the exposure of production workers. As a consequence, exposure is assumed to be limited to relatively low levels and accordingly only very small quantities of biomarkers of exposure are expected to

be present in any biological matrix of the worker. From limited research performed in our laboratory involving urine samples from patients receiving transdermal fentanyl in the treatment of cancer pain, it was estimated that the concentration of the urinary biomarkers of opioid exposed workers would lie several orders of magnitude below the urinary amounts of fentanyl and norfentanyl encountered in the therapeutic range. Consequently, analytical procedures presented in the literature for the determination of fentanyl-like compounds and their metabolites in pharmacokinetic studies will likely suffer from a lack of sensitivity. Therefore, the purpose of this study was to develop and comprehensively validate a highly sensitive gas chromatographic–mass spectrometric analytical method to determine picogram amounts of fentanyl, sufentanil and alfentanil and their major metabolites in urine of potentially exposed opioid production workers. Emphasis was further placed on a simple and rapid SPE isolation of the compounds of interest and the overall need for a fast and high-throughput biomarker screening assay. Recently, the newly developed method was successfully applied to measure the concentration levels of the proposed biomarkers in urine of workers occupationally exposed to fentanyl related compounds.

2. Experimental

2.1. Chemicals and materials

Fentanyl citrate, alfentanil hydrochloride, sufentanil citrate and the internal standard analogues d_5-fentanyl citrate, d_5-alfentanil hydrochloride and d_5-sufentanil citrate were kindly provided by Janssen Pharmaceutica (Beerse, Belgium). Norfentanyl and norsufentanil (noralfentanil) were also obtained from Janssen Pharmaceutica (Beerse, Belgium). The internal standard analogue d_5-norfentanyl (100 μg/ml, 99%) was supplied by LGC Promochem Sarl (Molsheim Cedex, France). *n*-Heptane (HPLC grade), *iso*-amylalcohol (anhydrous), acetone (HPLC grade), acetonitril (HPLC grade), pentafluorobenzoylchloride (PFBCl) (99%), and poly(ethylene)glycol (average MN 200) were obtained from Sigma–Aldrich (Steinheim, Germany). Toluene (p.a.) and disposable 15 ml sample tubes (16 mm × 100 mm) were supplied by VWR (Heverlee, Belgium). Methanol (HPLC grade) was obtained from Fisher Chemicals (Leicester, UK). Merck (Darmstadt, Germany) supplied the 1 ml EXtrelut® NT1 and 3 ml EXtrelut® NT3 Solid Phase Extraction columns. Autosampler vials (2 ml, crimp cap) were obtained from Machery–Nagel (Düren, Germany). Dimethyldichlorosilane, glass conical inserts (100 μl) and self-centering supports were supplied by Supelco (Bellefonte, USA).

2.2. Instrumentation and chromatographic conditions

The analyses were carried out on a Hewlett-Packard 6890 series gas chromatograph equipped with an autosampler and a 5973 series mass selective detector (MSD) in electron impact (EI) mode (70 eV). For the determination of the opioid narcotics, a 5 μl aliquot of the sample was introduced in a splitless way onto a DB35-MS (J&W) column with a nominal length of 30 m, an internal diameter of 0.25 mm and a film thickness of 0.15 μm. For the analysis of the nor-metabolites, a 30 m DB5-MS (J&W) column (0.25 mm i.d., 0.1 μm film) was used. A constant high purity Helium flow of 2.5 ml/min was applied through the columns. The GC separation was obtained using a program with an initial oven temperature of 70 °C that was increased at a rate of 60 °C/min to a final temperature of 280 °C. The oven was held at the final temperature for an additional 5.0 min. The injector and MS source temperature were maintained at 230 °C. The MS quadrupole temperature was held at 150 °C. The mass selective detection system was operated in the selected ion monitoring (SIM) mode. Base ion fragments occurring at *m/z* 245 for fentanyl, and *m/z* 250 for d_5-fentanyl, *m/z* 289 for sufentanil and alfentanil and *m/z* 294 for d_5-sufentanil and d_5-alfentanil were monitored and used for subsequent quantification. The nor-metabolite pentafluorobenzamide (PFBA) derivatives were monitored using their specific molecular ion fragments at *m/z* 426 for norfentanyl-PFBA, *m/z* 425 for norsu(al)fentanil-PFBA and *m/z* 431 for the internal standard analogue d_5-norfentanyl-PFBA. Individual ion dwell times were set at 100 ms for the opioid narcotics and their metabolites and at 25 ms for the deuterated internal standard analogues.

2.3. Preparation of standard solutions

It should be noted that all concentrations mentioned in this paper refer to the free base. Stock standard solutions of fentanyl and sufentanil (0.1 mg/ml) and of alfentanil, norfentanyl and norsu(al)fentanil (1 mg/ml) were prepared in methanol from the respective pure chemicals. Chemical purity of the analytes was determined by chromatographic analysis and was found to be 97.6, 99.2, 99.9, 96.6 and 90.0%, respectively. A working standard solution was prepared by appropriate dilution of the stock solution in isotone saline. An internal standard solution was prepared containing d_5-fentanyl citrate, d_5-alfentanil hydrochloride, d_5-sufentanil citrate and d_5-norfentanyl at final concentrations of 0.3 μg/ml for the deuterated parent compounds and 1 μg/ml for the metabolite internal standard.

2.4. Preparation of urine calibrators and QC samples

Blank urine was obtained from healthy adult volunteers at our department who were not involved in the fentanyl research described. Urine calibrators were prepared by adding microliter quantities of the working standard solution to 25 ml aliquots of blank urine. The concentration of these urine calibrators ranged from 5 to 150 pg fentanyl

and sufentanil per ml of urine, 25 to 750 pg/ml of alfentanil and 50 to 1500 pg/ml of both nor-metabolites. Accordingly, Quality Control (QC) samples were prepared at intermediate concentration levels. Individual concentration levels were selected to reflect the parent compounds' relative potency and hence their corresponding acceptable levels in the working environment on the one hand, and to simulate expected actual urine sample composition concerning the relative excess of nor-metabolites on the other hand.

2.5. Solid phase extraction of urine standards and samples

Aliquots of 1 ml of each urine standard and sample were pipetted into a disposable 15 ml sample tube. The samples were basified with 40 μl of 10N NaOH and 50 μl of the internal standard solution containing the deuterated analogues was added. The samples were applied to a 1 ml EXtrelut® NT1 SPE column. During 10 min the samples were allowed to spread over the chemically inert matrix of the SPE column. Elution was then carried out using 6 ml of a mixture of *n*-heptane/*iso*-amylalcohol (98.5/1.5 v/v). The extracts were evaporated at 50 °C using a gentle stream of nitrogen. For the determination of the parent opioid compounds, cooled residues were reconstituted in 30 μl of methanol containing 0.01% (v/v) poly(ethylene)glycol. For the determination of the opioid nor-metabolites, the cooled residues were derivatized with pentafluorobenzoylchloride using the protocol described in Section 2.6. All samples were sonicated for 5 minutes and transferred into a 100 μl glass conical insert containing autosampler vial and analyzed.

2.6. Derivatization of urine standards and samples

Derivatization of the secondary amine functionalities of the nor-metabolites norfentanyl and norsu(al)fentanil and the internal standard d_5-norfentanyl was carried out following the procedure of Valaer et al. [21] with some modifications. Briefly, 100 μl of a 0.1 M pentafluorobenzoyl chloride solution in chloroform was added to the residues but no heating was applied. Instead, the residues were allowed to react overnight (16 h) at 4 °C. After that period, the samples were dried under a gentle stream of nitrogen and the residues were reconstituted in 30 μl of acetonitril.

3. Results

Initial validation studies were performed using optimum sample preparation and analytical parameters for the opioid parent compounds on the one hand and their nor-metabolites on the other. Unless otherwise stated, the results presented refer to the individual assays. In the final phase of the study the feasibility of developing one sample preparation protocol and analytical procedure for the simultaneous determination of all analytes of interest was examined.

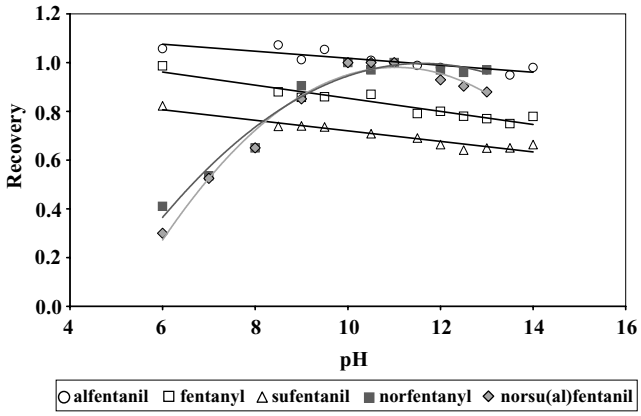

Fig. 2. Influence of pH of the urine samples on the extraction recovery of the analytes using SPE.

3.1. Sample preparation

A simple one-step SPE extraction procedure was applied in which commercially available, ready to use columns were loaded and eluted under hydrostatic pressure, resulting in clean biological extracts. One of the major improvements over existing SPE procedures was a redundancy of several column pre-cleaning, pre-conditioning and washing steps which are labor-intensive and solvent consuming and can only be achieved in an acceptable sample preparation time by the use of vacuum technology. Applying the procedure described, up to 150 samples can be prepared in a single SPE run.

The influence of pH of the urine samples on the recovery of all analytes using the developed SPE procedure was determined in a range of pH 6–14. Recovery was expressed as the ratio of mean (relative) peak areas ($n = 3$) of the analytes extracted from urine to the mean (relative) peak areas ($n = 3$) of extracted blank urine spiked post extraction. Recovery was evaluated at a fixed concentration using urine calibrator QC-3. Fig. 2 graphically presents the recovery of all analytes as a function of urinary pH. For the opioid parent compounds a downward tendency was observed of the analyte recovery as a function of urinary pH. Linear regression analysis showed an average decrease in recovery of 1.4% (alfentanil) to 2.7% (fentanyl) per unit increase of pH in the range studied. The optimum extraction pH was situated at pH 6 at which a recovery of alfentanil, fentanyl and sufentanil of 100, 99 and 82% was obtained. The recovery of norfentanyl and norsufentanil on the other hand was considerably lower at pH 6 and seemed to be optimal in a urinary pH range of 10–13, decreasing at both lower and higher pH values. For simultaneous extraction of all analytes from urine, a compromise in optimum recovery seemed to be reached at pH 11. However, adding a fixed amount of 40 μl 10N NaOH resulting in a pH >13 was preferred, as no monitoring of the original or newly obtained urinary pH was necessary and the resulting decrease in recovery was shown to be negligible.

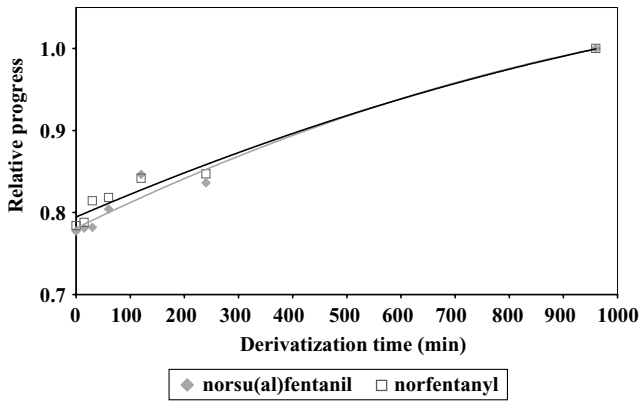

Fig. 3. Relative progress in time of the completeness of the derivatization procedure to yield PFBA-derivatives of the nor-metabolites of the opioid narcotics.

3.2. Derivatization

The nor-metabolites of the opioid narcotics contain a basic secondary nitrogen that can be derivatized with pentafluorobenzoylchloride (PFBCl) to form stable pentafluorobenzamide (PFBA)-products with good chromatographic properties.

The progress in the degree of PFBCl-derivatization of the nor-metabolites in spiked urine samples was monitored through the formation of the respective PFBA-derivatives at different time intervals, ranging from 5 min to 16 h. At each time interval three replicate QC-5 samples containing 1000 pg/ml of the nor-metabolites were processed and analyzed. Completeness of the derivatization step was expressed as a fraction of the PFBA-derivatives formed at a specific time, to those found in the 16 h samples. The results are presented graphically in Fig. 3. The relative progress of the PFBCl-derivatization was found to be at least 80% immediately after induction, steadily increasing over time and was estimated to be complete at 16 h (960 min) for both nor-metabolites.

3.3. Specificity

Under the chromatographic conditions described in the experimental section, all analytes of interest were well separated on the GC–MS chromatogram. For the opioid narcotics and norsu(al)fentanil, no significant interference was observed in the blank urine samples at the retention time of the compounds. Representative chromatograms of blank urine samples and urine samples fortified with all compounds at the intermediate QC level (QC-3) are shown in Figs. 4–7. The retention times for fentanyl, sufentanil and alfentanil were 6.30, 6.76 and 9.18 min. respectively. The retention times for the PFBA-derivatives of norfentanyl and norsu(al)fentanil were 5.23 and 5.41 min, respectively. For the derivative of norfentanyl a small interference in blank urine was observed at the retention time of the analyte

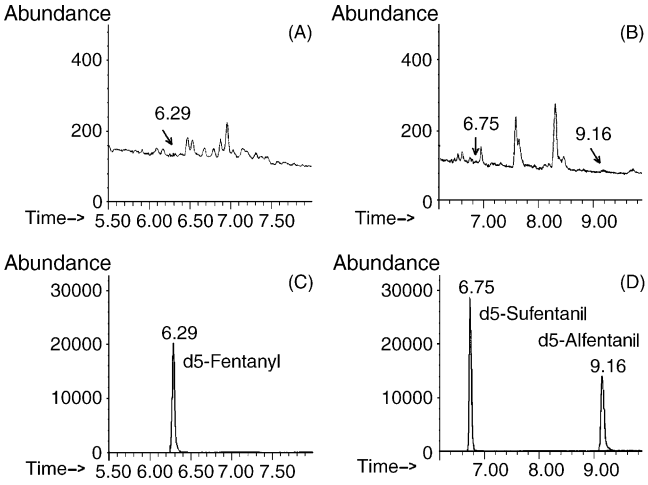

Fig. 4. Example chromatogram of an extracted blank urine sample showing peaks at *m/z* 250 (C) and *m/z* 294 (D) from the internal standards d_5-fentanyl and d_5-alfentanil and d_5-sufentanil respectively. At their respective retention times, no significant interference is observed at the ion fragments *m/z* 245 (A) and *m/z* 289 (B) monitored for the opioid narcotics.

(Fig. 6). This small interfering peak at *m/z* 426 is presumably partly due to a small fraction of non-deuterated norfentanyl in the deuterated internal standard solution. Adding increasing amounts of internal standard resulted in a larger interference, although this increase was not proportional.

3.4. Linearity

Six level calibration curves for the opioid parent compounds and their major metabolites were obtained by plotting the peak area ratio of the quantification ion of the analyte and its respective deuterated internal standard against the corresponding concentrations of the analyte in

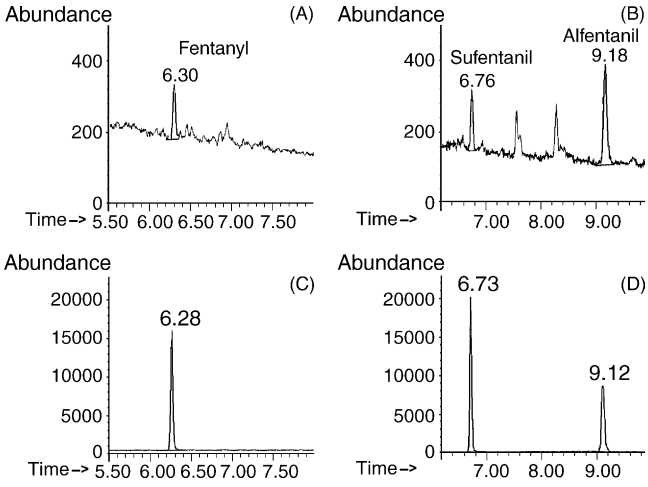

Fig. 5. Example chromatogram of a QC urine sample spiked with the opioid narcotics at the intermediate QC-level (QC-3). Ion fragments monitored for the opioid narcotics (*m/z* 245 and 289) (A, B) as well as those monitored for the deuterated analogues (*m/z* 250 and *m/z* 294) (C, D) are shown.

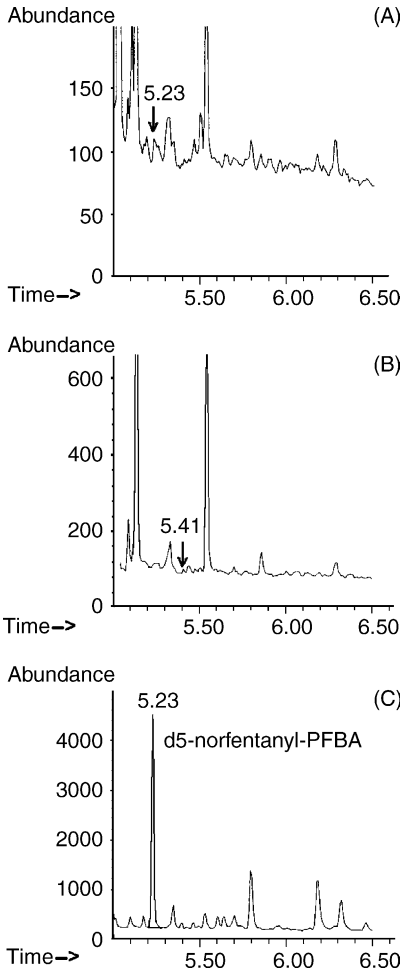

Fig. 6. Example chromatogram of an extracted blank urine sample showing peaks at *m/z* 431 (C) from the internal standard d_5-norfentanyl-PFBA derivative. No significant interference is observed at the ion fragment *m/z* 425 (B) monitored for the norsu(al)fentanil-PFBA derivative. At the retention time of norfentanyl-PFBA, a small interference at *m/z* 426 (A) is observed.

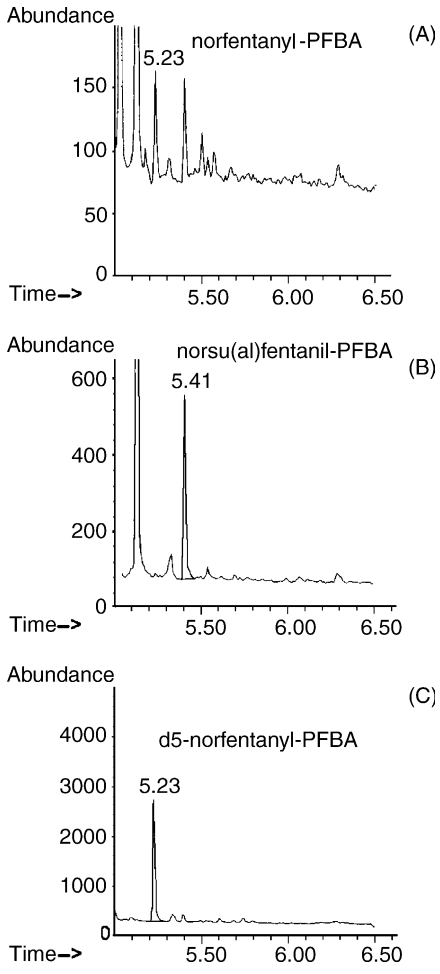

Fig. 7. Example chromatogram of a QC urine sample spiked with the opioid metabolites at the intermediate QC-level (QC-3). Ion fragments monitored for the opioid metabolite PFBA-derivatives of norsu(al)fentanil and norfentanyl (*m/z* 425 and 426, respectively) as well as those monitored for the deuterated internal standard derivative (*m/z* 431) are shown.

the urine calibrators. At each calibration level three replicate calibrator samples were analyzed. Linear regression analysis of the calibration plots resulted in the equations and correlation coefficients listed in Table 1. The analytical procedures showed good linearity over the entire ranges measured. For alfentanil a significant but analytically irrelevant negative intercept was observed, probably caused by a minor shift in the slope of the regression curve due to slightly raised data points at the end of the curve. For norfentanyl a significant positive intercept was observed, again indicating a small interference present in processed urine samples.

3.5. Limits-of-detection (LODs)

It was observed that in order to continuously achieve the desired sensitivity at these ultra low concentration levels several factors needed consideration. It was shown to be of

Table 1
Coefficients of the linear regression analysis ($Y = aX + b$) on the six level calibration curves for the opioids and their metabolites in urine

	Slope (95% CI)	Intercept (95% CI)	Intercept (*P*-value)	R^2
Fentanyl	0.162 (0.158–0.167)	1.6E−05 (−3.5E−04 to 3.9E−04)	0.93	0.997
Sufentanil	0.126 (0.122–0.129)	2.3E−04 (−4.3E−05 to 5.0E−4)	0.093	0.997
Alfentanil	0.099 (0.098–0.101)	−7.5E−04 (−1.3E−03 to −2.4E−04)	0.0059	0.999
Norfentanyl	0.025 (0.020–0.030)	8.6E−03 (5.3E−03 to 1.2E−02)	0.0007	0.963
Norsu(al)fentanil	0.388 (0.371–0.401)	−7.9E−03 (−1.8E−02 to 2.3E−03)	0.12	0.994

X = ng analyte/ml. Y = peak area ratio *m/z* 245/250 (fentanyl), 289/294 (alfentanil and sufentanil), 426/431 (norfentanyl) and 425/431 (norsu(al)fentanil) and 95% CI: 95% confidence interval.

primary importance to change GC-inlet liners at least every 250 injections and to use inlet liners that were freshly silanized. The silanizing protocol involved immersion of the liner in a 5% dimethyldichlorosilane solution in toluene for 10 min, then rinsing in acetone, followed by toluene and oven drying at 100 °C. It should be noted however that in contrast with the procedure often stated in the literature [22–24] none of the other glass ware used was treated with a silanizing reagent. Adsorption of the analytes of interest to active sites of different glass materials used through the sample preparation protocol, was prevented by the addition of relatively large amounts of the deuterated analogues of the compounds as internal standards which competed favorably for active adsorption sites. For the same purpose, also a small percentage of poly(ethylene)glycol (0.01%, v/v) was incorporated in the final residual extract of the opioid narcotics.

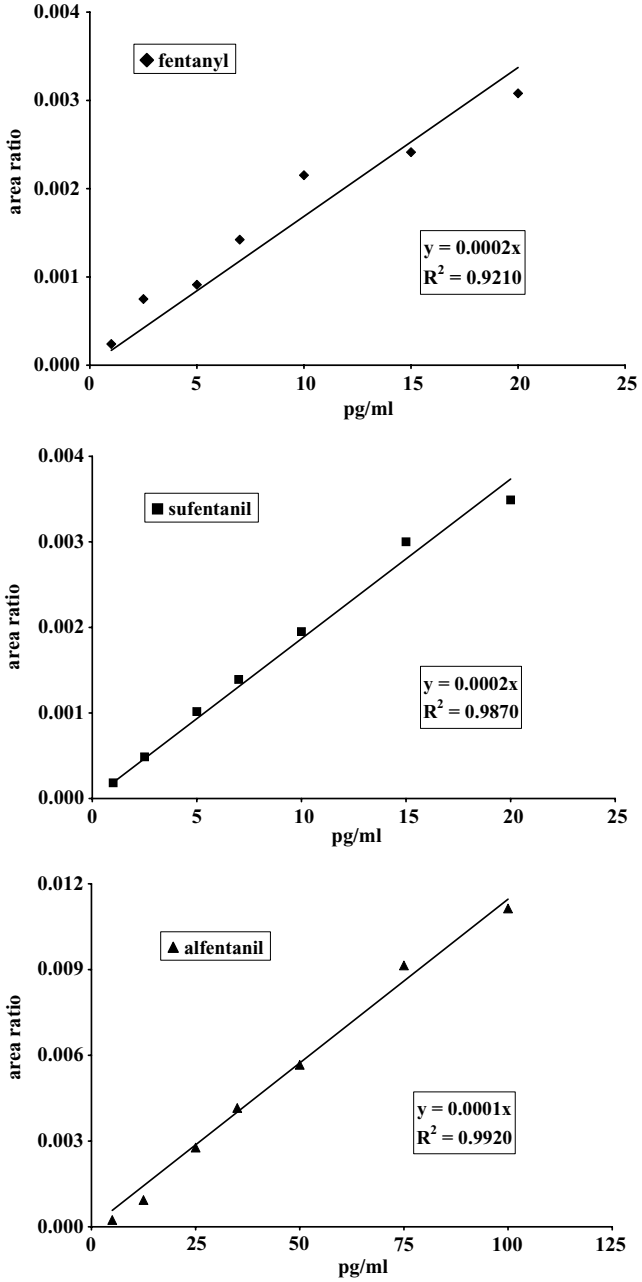

Fig. 8. Exploration of the urinary LOD for fentanyl, sufentanil and alfentanil. Additional linear regression curves of the opioid narcotics, obtained by plotting their respective peak area ratio against the ultra low concentration levels in urine (pg/ml).

Table 2
Analytical LOD of the opioid narcotics in urine samples, calculated as the average background ($n = 3$) shown in blank urine ± 3 S.D.

	LOD (blank ± 3 S.D.) (pg/ml)	LOD (*t*-test) (pg/ml)	R.S.D. at LOD ($n = 3$) (%)
Fentanyl	1.4	2.5	25
Sufentanil	2.8	2.5	18
Alfentanil	6.1	7.5	27
Norfentanyl	34.3	50.0	17
Norsu(al)fentanil	24.2	50.0	10

An alternative calculation for LODs included the identification of the calibrator level that showed a significant difference (*t*-test, $\alpha = 0.05$) in response as compared to the background level in blank urine. At that calibrator level, also the R.S.D. on triplicate analysis was calculated as a measure for precision.

To explore the sensitivity of the analytical procedures, an additional calibration curve was established for the opioid narcotics in the ultra low range of 1–20 pg/ml for fentanyl and sufentanil and in a range of 5–100 pg/ml for alfentanil. Again, good or excellent linearity was observed even at these extremely low levels (Fig. 8). The limit-of-detection (LOD), in terms of the lowest detectable amount of the opioids, was calculated as the average background ($n = 3$) shown in blank urine ± 3 standard deviation (S.D.). For the opioid metabolites a similar calculation was performed using the lowest calibrator levels prepared for the linearity test. An alternative calculation for LODs of all compounds included the identification of the calibrator level that showed a significant difference (*t*-test, $\alpha = 0.05$) in response as compared to the background level in blank urine. At that calibrator level, also the precision, expressed as relative standard deviation (R.S.D.) on triplicate analyses, was calculated. The resulting LODs and R.S.D.s are presented in Table 2. In conclusion, the sensitivity of the analytical procedures, expressed as the LOD was found to be <5 pg/ml for fentanyl and sufentanil, <10 pg/ml for alfentanil and <50 pg/ml for the nor-metabolites. Even at these low compound levels the reproducibility is still sufficient with a coefficient of variation ranging from 10 to 27%.

3.6. Intra-assay accuracy and precision

The analytical intra-assay precision of the biomonitoring procedures was defined as the coefficient of variation (CV) resulting from the analysis of a set of six replicate urine samples spiked with various concentrations of the opioid narcotics corresponding to the QC calibrators. The accuracy was determined by comparing the means of measured concentrations with the nominal concentration for the same QC

Table 3
Analytical intra-assay precision expressed as the coefficient of variation (CV) and accuracy expressed as RE ($n = 6$) for the opioid narcotics and their metabolites spiked in urine at the QC calibrator levels

	Intra-assay precision and accuracy ($n = 6$)				
	QC-1	QC-2	QC-3	QC-4	QC-5
Fentanyl					
Added (pg/ml)	10.0	25.0	50.0	75.0	100.0
Mean ± S.D. (pg/ml)	9.0 ± 0.74	22.6 ± 1.7	53.8 ± 3.7	77.3 ± 4.5	105.2 ± 6.0
CV (%)	8.2	7.6	6.8	5.8	5.7
RE (%)	−9.9	−9.7	7.7	3.1	5.2
Sufentanil					
Added (pg/ml)	10.0	25.0	50.0	75.0	100.0
Mean ± S.D. (pg/ml)	13.0 ± 1.2	25.5 ± 1.2	57.6 ± 2.8	78.4 ± 3.2	102.5 ± 4.1
CV (%)	8.9	4.6	4.9	4.0	4.0
RE (%)	29.8	2.1	15.2	4.6	2.5
Alfentanil					
Added (pg/ml)	50.0	125.0	250.0	375.0	500.0
Mean ± S.D. (pg/ml)	48.9 ± 2.7	115.8 ± 8.2	271.0 ± 8.4	382.9 ± 8.3	523.4 ± 26.9
CV (%)	5.5	7.0	3.1	2.2	5.1
RE (%)	−2.3	−7.4	8.4	2.1	4.7
Norfentanyl					
Added (pg/ml)	100.0	250.0	500.0	750.0	1000.0
Mean ± S.D. (pg/ml)	118.3 ± 61.5	237.3 ± 50.7	533.0 ± 60.3	736.9 ± 41.1	936.2 ± 67.9
CV (%)	52.1	21.4	11.3	5.6	7.3
RE (%)	18.1	−5.1	6.6	−1.7	−6.4
Norsu(al)fentanil					
Added (pg/ml)	100.0	250.0	500.0	750.0	1000.0
Mean ± S.D. (pg/ml)	101.5 ± 8.3	227.8 ± 13.7	514.5 ± 10.8	738.7 ± 35.8	1023.1 ± 30.0
CV (%)	8.2	6.0	2.1	4.8	2.9
RE (%)	1.5	−8.9	2.9	−1.5	2.3

calibrators. Intra-assay precision data and accuracy of the analytical procedure for the opioids and their nor-metabolites are presented in Table 3. Coefficients of variations were below 10% at all urinary spike levels of fentanyl, sufentanil and alfentanil. Accuracy, expressed as relative error (RE), was determined to be below 10% for all opioids at all spike levels, except for sufentanil at the lowest spike level (29.8%) and at the intermediate QC level (15.2%). For norsu(al)fentanil coefficients of variation and relative error were below 10% at all urinary spike levels. Relative error was below 10% for norfentanyl at all spike levels, except for the lowest spike level (18.1%). Norfentanyl precision data were less opportune at the lowest spike levels (52.1 and 21.4%) improving to less than 10% at the highest spike levels. These higher coefficients of variation presumably were due to the presence of a minor interference, which affected particularly the precision at low norfentanyl levels.

To improve the precision of the norfentanyl assay, limited tests were performed using 3 ml urine samples, fortified with the internal standard solution and subjected to a slightly modified sample preparation protocol. Briefly, the SPE-extraction protocol involved loading of the basified urine sample on a 3 ml EXtrelut® NT3 SPE column, constituting a similar extraction matrix as the 1 ml EXtrelut® NT1 columns. Elution was carried out using 15 ml of a mixture of *n*-heptane/*iso*-amylalcohol (98.5/1.5 v/v). The extracts were evaporated at 50 °C using a gentle stream of nitrogen and the cooled residues were derivatized with PFBCl using the protocol described in Section 2.6. The intra-assay precision and accuracy data are presented in Table 4. For all

Table 4
Analytical intra-assay precision expressed as the coefficient of variation (CV) and accuracy expressed as RE ($n = 3$) for norfentanyl spiked in urine at the QC calibrator levels, using a slightly modified 3 ml urine sample extraction protocol

	Intra-assay precision and accuracy ($n = 3$)				
	QC-1	QC-2	QC-3	QC-4	QC-5
Norfentanyl					
Added (pg/ml)	100.0	250.0	500.0	750.0	1000.0
Mean ± S.D. (pg/ml)	100.7 ± 20.6	235.6 ± 46.2	510.9 ± 45.9	775.7 ± 17.0	1009.7 ± 44.1
CV (%)	20.5	19.6	9.0	2.2	4.4
RE (%)	0.67	−5.8	2.2	3.4	1.0

norfentanyl spike levels CVs were lower as compared to the 1 ml urine sample preparation protocol, being especially advantageous at the lowest spike levels. The accuracy, expressed as RE, was generally comparable to those found with the 1 ml sample protocol. At the lowest norfentanyl spike level an exceptional low RE was observed, but in view of the limited number of test samples, this result should be interpreted with some reservations.

In summary, the developed analytical procedures show excellent accuracy, particularly considering the ultra low levels of the analytes, with relative errors generally below 10%. Overall, an excellent reproducibility was observed with coefficients of variation below 10% at all spike levels for all opioid parent compounds and for norsu(al)fentanil. However, at the lowest norfentanyl spike levels the precision of the analytical procedure was poor, although accuracy was quite acceptable.

3.7. Inter-assay accuracy and precision

The inter-assay precision of the analytical procedures was defined as the coefficient of variation resulting from the analysis of the QC level urine calibrators at 5 different time intervals over a period of approximately 3 months. The accuracy was determined by comparing the means of measured concentrations with the nominal concentration for the same QC calibrators. The data are presented in Table 5. Coefficients of variations (CV) are generally below 20%, except for the lowest spike level of fentanyl (24.5%) and sufentanil (31.4%). Precision of the assay was characterized by CVs generally below 10%, except for the lowest norfentanyl spike levels. Accuracy, expressed as relative error, was below 15% for all opioids and both metabolites all spike levels. Considering the ultra low levels of the opioids and their metabolites, the analytical procedures demonstrate an excellent accuracy and an acceptable reproducibility over time.

3.8. Stability of urine samples during storage

To evaluate the stability of the opioid narcotics and their metabolites during storage of urine samples, various experiments were set up. For the opioid narcotics, four sets of urine samples were spiked with individual analyte concentrations ranging from 20 to 100 pg/ml for fentanyl and sufentanil and ranging from 100 to 500 pg/ml urine for alfentanil. One set of spiked urine samples was analyzed immediately. Three other sets of spiked samples were stored at −30 °C during 1, 2 and 3 months respectively and subsequently analyzed. At each storage period, recoveries of the opioid narcotics were compared to those found in non-stored samples. For the opioid metabolites similar tests were performed, using urine calibrators in a concentration range of 200–5000 pg/ml. For

Table 5
Analytical inter-assay precision expressed as the coefficient of variation (CV) and accuracy expressed as RE ($n = 5$) for the opioid narcotics and their metabolites spiked in urine at the QC calibrator levels

	Inter-assay precision and accuracy ($n = 5$)				
	QC-1	QC-2	QC-3	QC-4	QC-5
Fentanyl					
Added (pg/ml)	10.0	25.0	50.0	75.0	100.0
Mean ± S.D. (pg/ml)	11.4 ± 2.8	27.7 ± 3.0	46.3 ± 4.2	81.5 ± 5.0	96.0 ± 4.3
CV (%)	24.5	10.7	9.0	6.2	4.5
RE (%)	14.4	10.9	−7.3	8.7	−4.0
Sufentanil					
Added (pg/ml)	10.0	25.0	50.0	75.0	100.0
Mean ± S.D. (pg/ml)	11.0 ± 3.4	26.3 ± 5.0	48.8 ± 5.4	82.5 ± 1.7	94.3 ± 4.1
CV (%)	31.4	19.1	11.0	2.0	4.3
RE (%)	9.8	5.3	−2.4	10.0	−5.7
Alfentanil					
Added (pg/ml)	50.0	125.0	250.0	375.0	500.0
Mean ± S.D. (pg/ml)	55.9 ± 4.1	122.8 ± 13.7	240.7 ± 20.9	375.7 ± 26.3	492.6 ± 32.2
CV (%)	7.3	11.1	8.7	7.0	6.5
RE (%)	11.9	−1.8	−3.7	0.20	−1.5
Norfentanyl					
Added (pg/ml)	100.0	250.0	500.0	750.0	1000.0
Mean ± S.D. (pg/ml)	107.6 ± 23.6	244.2 ± 62.8	533.6 ± 48.8	837.4 ± 71.5	922.8 ± 15.6
CV (%)	21.9	25.7	9.1	8.5	1.7
RE (%)	7.6	−2.3	6.7	11.7	−7.7
Norsu(al)fentanil					
Added (pg/ml)	100.0	250.0	500.0	750.0	1000.0
Mean ± S.D. (pg/ml)	109.1 ± 10.7	232.6 ± 6.8	521.0 ± 9.1	783.2 ± 62.9	977.3 ± 64.7
CV (%)	9.8	2.9	1.8	8.0	6.6
RE (%)	9.1	−7.0	4.2	4.4	−2.3

Table 6
Stability of the opioid narcotics and their major metabolites in urinary calibrators stored at −30 °C during 1, 2 and 3 months

	Average recovery ± S.D. ($n = 5$) upon storage		
	At 1 month	At 2 months	At 3 months
Fentanyl	0.93 ± 0.05	1.03 ± 0.07	1.09 ± 1.0
Sufentanil	0.96 ± 0.05	0.99 ± 0.09	0.93 ± 0.01
Alfentanil	1.02 ± 0.02	1.12 ± 0.09	0.77 ± 0.05
Norfentanyl	NT	1.14 ± 0.16	0.95 ± 0.14
Norsu(al)fentanil	NT	NT	1.07 ± 0.12

At each storage period, recoveries were compared to those found in non-stored samples and were averaged over the entire concentration range studied ($n = 5$). NT: not tested.

norfentanyl two storage periods were considered (2 and 3 months), while for norsu(al)fentanil storage stability was evaluated after the total period of 3 months. The data in Table 6 show the analyte recoveries at the different storage periods, averaged ($n = 5$) over their entire individual concentration range studied. No significant loss seemed to occur of either the opioid narcotics or norfentanyl upon storage of spiked urine samples during 1 or 2 months. Urinary calibrator samples containing either fentanyl, norfentanyl or norsu(al)fentanil were found to be stable at −30 °C for at least 3 months. At this storage condition a small (7%) but significant loss seemed to occur for sufentanil (*t*-test, $P = 0.024$), being significantly more important (23%) for alfentanil (*t*-test, $P = 0.0006$).

3.9. Simultaneous determination of the opioid parent compounds and their metabolites

In order to examine the feasibility of developing one sample preparation protocol and analytical procedure for the simultaneous determination of all analytes of interest, several factors needed consideration.

The chromatographic separation of the opioid narcotics and their nor-metabolites was evaluated using a standard solution containing fentanyl and sufentanil in a final concentration of 100 μg/ml and alfentanil and the opioid metabolites in a final concentration of 300 μg/ml methanol. After evaporation, the solution was derivatized according to the previously described protocols. A 2 μl aliquot of the sample was introduced in a splitless way onto a DB35-MS (J&W) column. Other chromatographic parameters were applied as mentioned in the instrumentation section. As shown in Fig. 9, chromatographic separation displayed was sufficient to allow the analysis of samples that might contain a mixture of the opioid narcotics and their metabolites.

The sample preparation protocol, previously developed for the opioid narcotics, was similar to the procedure used for their nor-metabolites, except for an additional derivatization step applied for the latter compounds. Therefore, the potentially adverse effect of the derivatization protocol on the recovery and stability of extracted opioid parent compounds was explored. Urinary test samples were prepared containing 20–1000 pg/ml fentanyl and sufentanil and 100–5000 pg/ml alfentanil. The urine samples were subjected to the SPE-extraction and evaporation step described above. At each concentration level one set of 6 replicate urinary extracts was reconstituted in methanol as described before and one set of three extracted replicates was subjected to the derivatization procedure indicated in 2.6. Recovery was determined by comparing the absolute areas of the quantification ion of the opioid compounds in both procedures. Recoveries of the individual opioids in the derivatized samples were averaged ($n = 18$) over all concentration levels studied and were determined to be 50 ± 8, 53 ± 8 and 38 ± 1% for fentanyl, sufentanil and alfentanil respectively. Recoveries of the deuterated internal standard analogues in the derivatized samples were comparable to those of the non-deuterated, as expected. As a consequence, peak area ratio's of parent compounds and deuterated analogues were similar for both procedures, resulting in comparable regression calibration curves. However, the lower absolute peak areas affected the analytical sensitivity of the method, increasing the LOD by at least a twofold.

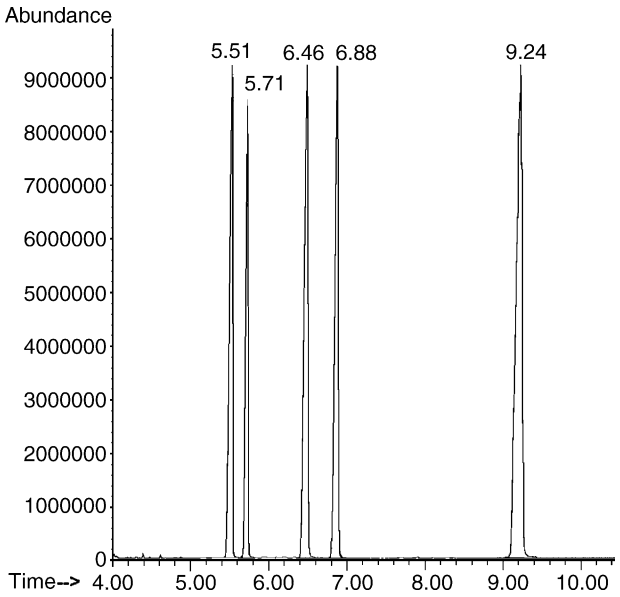

Fig. 9. Full scan GC–MS chromatogram of a derivatized standard solution containing fentanyl and sufentanil in a final concentration of 100 μg/ml and alfentanil and the opioid metabolites in a final concentration of 300 μg/ml methanol. Adequate separation of all analytes of interest was obtained. The respective retention times were 5.51 min (norfentanyl), 5.71 min (norsu(al)fentanil), 6.46 min (fentanyl), 6.88 min (sufentanil) and 9.24 min (alfentanil).

3.10. Application

The developed GC–MS analytical procedures have been recently used in a biological monitoring survey to quantitate fentanyl and norfentanyl in urine of potentially opioid exposed production workers. Urine samples were collected

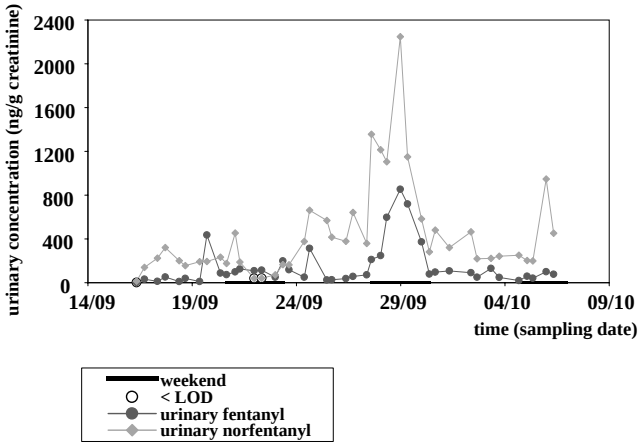

Fig. 10. Fentanyl concentrations (●) and norfentanyl concentrations (◆) (ng/g creatinine) measured in the urine of a production worker at different time intervals during a 3 weeks production campaign. Measurements below the LOD (2.5 pg fentanyl/ml and <50 pg norfentanyl/ml) are indicated as '○'. Weekends are placed in bold on the *X*-axis.

at different time intervals during a 3 weeks fentanyl production campaign. Fig. 10 shows the fentanyl and norfentanyl urinary concentration profile as a function of time determined in an elective production worker. For data-analysis purposes all urinary concentrations were corrected for urinary creatinine content. It should be noted however that one sample with a urinary creatinine concentration below 0.3 g/l was not considered valid. The first urine sample, collected before fentanyl production was started, showed no detectable amount of fentanyl or norfentanyl ('○'). All subsequently collected urine samples had detectable amounts of fentanyl, ranging from 7.5 to 900 pg/ml urine (10–854 ng fentanyl/g creatinine). Correspondingly, all subsequently collected samples except for two showed detectable amounts of norfentanyl ranging from 75 to 2175 pg/ml urine (72–2247 ng norfentanyl/g creatinine). These data indicate that the developed analytical procedures show adequate sensitivity for the biological monitoring of opioid production workers. Although exploratory correlation analysis of urinary fentanyl and norfentanyl concentration levels revealed a significant correlation (Pearson $r = 0.7$, $P < 0.005$) between both biomarkers in this worker, an extended discussion of this promising finding falls beyond the purpose of this paper and will be discussed in length elsewhere. As was observed from the concentration versus time plots in Fig. 10, urine samples collected during the weekends (indicated in bold on the *X*-axis), unexpectedly also showed detectable fentanyl and norfentanyl levels. It was hypothesized that a delayed fentanyl clearance appeared to occur, possibly as a result of unintentional and prolonged exposure through contamination of the worker's skin. This intriguing hypothesis will be subject to further exploration during a follow-up study involving—amongst other parameters—the evaluation of dermal fentanyl exposure.

4. Discussion

In the present study, the development and validation procedures for sensitive GC–MS determination of the opioid narcotics and their major nor-metabolites in opioid exposed workers are described. In the initial phase of the study separate sample preparation protocols and analytical parameters were used for the opioid parent compounds on the one hand and their nor-metabolites on the other. The developed assays represent a substantial improvement in both analytical sensitivity and sample preparation protocol over methods reported in the literature that were developed primarily in the scope of pharmacokinetic studies. Historically, these techniques were based on immunological principles and include various radioimmuno assays (RIA). Although recently researchers have been very successful in developing novel and sensitive immunoassays [25,26], routine immunological procedures tend to suffer from cross-interference [27,28] and have quantitative cut off points in the order of 0.1 ng of the active substance per ml of biological matrix [22,29–32]. During the last decade, instrumental analyses, including both liquid and gas chromatographic separations with diverse detection systems, have been developed and validated, focussing on the identification and quantification of the fentanyl-like compounds and their metabolites, mainly in human plasma. Chromatographic assays using mass-selective detection procedures are highly specific and accordingly sensitive but in general limits of quantification of 20–300 pg/ml are reported, [21–23,28,33–35] which were suspected to be still unsatisfactory for the present study purpose. In our newly developed analytical procedures, only 1 ml of urine was required to achieve limits of detection (LODs) of 2.5 pg fentanyl/ml, 2.5 pg sufentanil/ml and 7.5 pg alfentanil/ml. For the opioid metabolites, which were analyzed as their PFBA-derivatives, the LODs were found to be <50 pg/ml urine. The developed analytical procedures show excellent intra-assay accuracy, particularly considering the ultra low levels of the analytes, with relative errors generally below 10%. Overall, an excellent reproducibility was observed with coefficients of variation below 10% at all spike levels for all opioid parent compounds and their metabolites, except for low norfentanyl concentrations.

Among the extraction techniques used to isolate the opioids from biological matrices, liquid–liquid extraction is by far the most frequently used. Although the use of solid-phase extraction (SPE) has grown dramatically over the last 10 years, this is not reflected in the number of reports related to the determination of fentanyl and analogues in biological matrices. In a few recent studies, this promising sample preparation technique has been applied to isolate fentanyl and sufentanil from human plasma [23,26,34,35]. One of the major disadvantages of the type of SPE reported in these studies is the need for several pre-cleaning, pre-conditioning, washing and eluting steps which are time and solvent consuming. In the present study, a simple one-step SPE

extraction procedure was developed in which commercially available, ready to use columns were loaded and eluted under hydrostatic pressure, resulting in clean biological extracts.

In the last stage of the study, the feasibility of developing one sample preparation protocol and analytical procedure for the simultaneous determination of all analytes of interest, was explored. Chromatographic separation displayed was sufficient to allow the analysis of samples that might contain a mixture of the opioid narcotics and their metabolites. However, the derivatization protocol applied to form PBBA-derivatives of the nor-metabolites, seemed to have a disadvantageous effect on the stability and recovery of the opioid parent compounds. Although peak area ratio's were found to be similar in both procedures, the lower absolute peak areas affected the analytical sensitivity of the method, increasing the LOD by at least a twofold. Additionally, the derivatization procedure also appeared to reduce the precision of the analytical method, determined as the coefficient of variation (CV) on peak area ratios of replicate sample extracts. Over the entire concentration range studied averaged precision coefficients of respectively 4.1, 6.1 and 4.0% were observed for fentanyl, sufentanil and alfentanil in the non-derivatized samples, while in the extracts subjected to the derivatization procedure CVs of respectively 8.7, 15.4 and 12.1% were calculated. Finally, the analysis of the derivatized urinary extracts resulted in less clean chromatograms, potentially complicating the chromatographic interpretation of subtle peaks displayed by low levels of the opioids. Taken into consideration the emphasis that was being laid on the desired sensitivity of the analytical methods, it was concluded that the disadvantageous effects of the derivatization protocol on the opioid recovery did not favor the development of a single sample preparation procedure in this study. However, the proposed single sample preparation and analysis protocol was successfully applied in pharmacokinetic studies in patients in which the opioid narcotics and their metabolites were simultaneously quantified in a ng/ml urine range.

In summary, highly sensitive analytical methods were developed and validated for the determination of the opioid narcotics fentanyl, sufentanil and alfentanil and their major metabolites in urine of exposed workers. Ongoing effort will be directed towards the feasibility of developing a single, common sample preparation protocol and analytical procedure for rapid and simultaneous screening of the proposed biomarkers of exposure in worker's urine samples. Furthermore, additional investigation is imperative to evaluate the significance of the different biomarker candidates in the assessment of occupational exposure to the opioid narcotics. Finally, the capability to monitor opioid metabolites in addition to the parent compounds may help clarify potential inter-individual differences in opioid metabolism and clearance.

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References

[1] L.E. Mather, Clin. Pharmacokinet. 8 (1983) 422.
[2] J.J. Downes, R.A. Kemp, C.J. Lambertsen, J. Pharmacol. Exp. Ther. 158 (1967) 416.
[3] W. Jeal, P. Benfield, Drugs 53 (1997) 109.
[4] S. Grond, L. Radbruch, K.A. Lehmann, Clin. Pharmacokinet. 38 (2000) 59.
[5] O. Dale, R. Hjortkjær, E.D. Kharasch, Acta Anaesthesiol. Scand. 46 (2002) 759.
[6] H. Zhang, J. Zhang, J.B. Streisand, Clin. Pharmacokinet. 41 (2002) 661.
[7] M.A. Clotz, M.C. Nahata, Clin. Pharm. 10 (1991) 581.
[8] J. Scholz, M. Steinfath, M. Schulz, Clin. Pharmacokinet. 31 (1996) 275.
[9] W. Meuldermans, K. Lavrijsen, J. Heykants, in: M. Chauvin (Ed.), Les morphiniques, Arnette S.A., Paris, 1993, p. 85.
[10] T. Goromaru, H. Matsuura, N. Yoshimura, T. Miyawaki, T. Sameshima, J. Miyao, T. Furuta, S. Baba, Anesthesiol. 61 (1984) 73.
[11] W. Meuldermans, A. Van Peer, J. Hendrickx, R. Woestenborghs, W. Lauwers, J. Heykants, G. Vanden Bussche, H. Van Craeyvelt, P. Van Der Aa, Anesthesiol. 65 (1988) 527.
[12] B.D. Naumann, E.V. Sargent, B.S. Starkman, W.J. Fraser, G.T. Becker, G.D. Kirk, Am. Ind. Hyg. Assoc. J. 57 (1996) 33.
[13] J.S. Willens, N.R. Myslinski, Heart Lung. 22 (1993) 239.
[14] P. Górski, S. Ulinski, Int. J. Occup. Med. Env. Health. 9 (1996) 245.
[15] W.A. Shaikh, Allergy. 45 (1990) 555.
[16] R.E. Biagini, D.M. Bernstein, S.L. Klincewicz, R. Mittman, I.L. Bernstein, G.M. Henningsen, J. Allergy Clin. Immunol. 89 (1992) 108.
[17] M.S. Morgan, Environ. Health Perspect. 105 (1997) 105.
[18] T. Stanley, B. Hague, D.L. Mock, J.B. Streisand, S. Bubbers, R.R. Dzelzkalns, P.L. Bailey, N.L. Pace, K.A. East, M.A. Ashburn, Anesth. Analg. 69 (1989) 21.
[19] R.B. Labroo, M.F. Paine, K.E. Thummel, E.D. Kharasch, Drug Metab. Dispos. 25 (1997) 1072.
[20] E.D. Kharasch, M. Russell, D. Mautz, K.E. Thummel, K.L. Kunze, A. Bowdle, K. Cox, Anesthesiol. 87 (1997) 36.
[21] A.K. Valaer, T. Huber, S.V. Andurkar, C.R. Clark, J. DeRuiter, J. Chromatogr. Sci. 35 (1997) 461.
[22] B. Fryirsa, A. Woodhouse, J.L. Huang, M. Dawson, L.E. Mather, J. Chromatogr. B. 688 (1997) 79.
[23] C. Dufresne, P. Favetta, C. Paradis, R. Boulieu, Clin. Chem. 47 (2001) 600.
[24] H.S. Choi, H.C. Shin, G. Khang, J.M. Rhee, H.B. Lee, J. Chromatogr. B. 765 (2001) 63.
[25] Y. Dotsikas, Y.L. Loukas, I. Siafaka, Anal. Chim. Acta. 459 (2002) 177.
[26] S.S. Rossi, J.B. Dyck, T.L. Yaksh, Clin. Chim. Acta. 244 (1996) 103.
[27] V.W. Watts, Y.H. Caplan, J. Anal. Toxicol. 14 (1990) 266.
[28] J.G. Schwartz, J.C. Garriot, J.S. Somerset, E.J. Igler, R. Rodriguez, M.D. Orr, Am. J. Forensic Med. Pathol. 15 (1994) 236.

[29] M. Michiels, R. Hendriks, J. Heykants, Eur. J. Clin. Pharmacol. 12 (1977) 153.
[30] M. Michiels, R. Hendriks, J. Heykants, J. Pharmacol. 35 (1983) 86.
[31] J.A. Phipps, M.A. Sabourin, W. Buckingham, L. Strunin, Can. Anaesth. Soc. J. 30 (1983) 162.
[32] J. Schüttler, P.F. White, Anesthesiol. 61 (1984) 315.
[33] D.S. Mautz, R. Labroo, E.D. Kharasch, J. Chromatogr. B. 658 (1994) 149.
[34] W.Z. Shou, X. Jiang, B.D. Beato, W. Naidong, Rapid Commun. Mass Spectrom. 15 (2001) 466.
[35] W. Naidong, H. Bu, Y.-L. Chen, W.Z. Shou, X. Jiang, T.D.J. Halls, J. Pharm. Biomed. Anal. 28 (2002) 1115.