Research Article

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Determination of fentanyl, metabolite and analogs in urine by GC/MS

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ABSTRACT: A rapid and sensitive method for the simultaneous determination of alfentanyl, sufentanyl and fentanyl (and its major metabolite norfentanyl) in urine was developed and validated. The method involved a liquid–liquid extraction in alkaline conditions, derivatization with pentafluoropropionic anhydride to improve the sensitivity for norfentanyl and subsequent analysis in GC/MS. The LODs are 0.08 ng ml^{-1} for all substances (0.04 ng ml^{-1} for alfentanyl). Intra- and inter-day precision coefficient of variation was always below 15%; mean relative error (accuracy) was always below 15%. The method was linear for all analytes, with quadratic regression of calibration curves always higher than 0.99. The method was applied to real samples of subjects who had received therapeutic doses of fentanyl, showing its suitability for the determination of low levels of these substances. The method was also applied to a subject whose death was attributed to fentanyl overdose. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: fentanyl and analogs; urine analysis; GC/MS; forensic toxicology; doping analysis

INTRODUCTION

Fentanyl is a powerful synthetic μ-opioid agonist, synthesized in 1960 and utilized in the treatment of chronic pain and in anesthesia (Gourlay *et al*., 1990; Mather, 1983). It is about 80 times more potent than morphine as analgesic and has a rapid onset and a short duration of action. In addition to fentanyl, other analogs have been synthesized, such as sufentanyl (more potent than fentanyl) and alfentanyl (with shorter action), with similar pharmacological characteristics (Lemmens, 1995; Scholz *et al*., 1996). Fentanyl can be delivered via transdermal patches, sublingual, nasal, rectal or intravenous administration. Fentanyl and analogs possess the same adverse effects as other opiates (vomiting, nauseas, fatigue, headaches, etc.; Porreca and Ossipov, 2009). Fentanyl is mainly metabolized through oxidative deamination at the piperidinic nitrogen to form the *N*-dealkylated norfentanyl (Goromaru *et al*., 1984).

In recent years, the illicit consumption of these substances has increased, often combined with other drugs of abuse or with alcohol, leading to an increasing number of fentanyl-related deaths (Denton *et al*., 2008; Hull *et al*., 2007; Kuhlman *et al*., 2003; Lilleng *et al*., 2004; Thompson *et al*., 2007; Wong *et al*., 2008). The determination of fentanyl, its main metabolite and its analogs is hence a main issue in forensic and clinical toxicology. Fentanyl and analogs are also included in the list of prohibited substances of the world antidoping agency (World Antidoping Agency, 2010a). The analytical determination of these substances is therefore also required in antidoping controls. Owing to their potent activity, therapeutic doses are generally very low (i.e. 0.1–1.2 mg for oral administration, or 12.5–100 μg h^{-1} for transdermal patches). Analytical methods must hence allow the determination of low concentrations of fentanyl and analogs. Methods described in the literature generally perform an SPE cartridge extraction for the determination of these substances (Goldberger *et al*., 2010; Gunnar *et al*., 2005; Poklis and Backer, 2004; Van Nimmen *et al*., 2004). Other methods involve the use of solid-phase microextraction prior to the analysis (Paradis et al., 2002; Bagheri *et al*., 2007). There are few studies that derivatize the extract prior to MS analysis (Hammergren and Henderson, 1988; Van Nimmen *et al*., 2004; Valaer *et al*., 1997). This paper describes a rapid and sensitive method for the simultaneous determination of alfentanyl, sufentanyl, fentanyl and its main metabolite norfentanyl in urine, by gas chromatography/mass spectrometry (GC/MS) after liquid–liquid extraction and derivatization of the nor metabolite, to form the pentafluoropropionic derivative, for its application in various fields of forensic toxicology.

MATERIALS AND METHODS

Chemicals and Reagents

Alfentanyl, fentanyl, norfentanyl, sufentanyl and fentanyl-D5 (internal standard, IS) were obtained from LGC Standards (Barcelona, Spain). Sodium hydroxide was purchased from Panreac (Castellar del Vallés, Spain); tert-butyl methyl ether and ethyl acetate were from Merck (Madrid, Spain). Pentafluoropropionic anhydride was from Aldrich, (Tres Cantos, Madrid, Spain)

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Preparation of Stock Positive Urines and Calibration Curves

Ten drug-free urines were obtained from laboratory staff and used for the preparation of calibration curves and for the repeatability and matrix effect studies.

Individual methanolic stock solutions containing 10 μg ml^{-1} of each of the listed standards were used to prepare the spiked urine at concentrations of 0.04, 0.08, 0.2, 0.5, 1, 5, 10, 25 and 50 ng ml^{-1} of fentanyl and analogs. Stock and working urine samples as well as methanolic standard solutions were stored at –20 °C until use.

Sample Preparation

First, 5 μl of internal standard (fentanyl-D5, 10 μg ml^{-1}) were added to 2 ml of urine. Afterwards, 150 μl of 2 M NaOH and 0.2 g of sodium chloride were added. Subsequently, a liquid–liquid extraction was performed with 5 ml of tert-buthyl methyl ether, centrifuged and the organic phase transferred and evaporated to dryness. The extract was then derivatised by the addition of 50 μl of pentafluoropropionic anhydride and 50 μl of ethyl acetate, and incubation at 70 °C for 20 min. The derivatised extract was then evaporated to dryness under nitrogen stream at 40 °C, and reconstituted into 50 μl of ethyl acetate three times to remove any anhydride residue. Finally, 20 μl of ethyl acetate were added and 1 μl was manually injected directly into the GC/MS.

GC/MS

GC/MS analyses were performed in an Agilent 6890 Gas Chromatograph coupled with an Agilent 5973 mass selective quadrupole detector (Agilent Technologies, Las Rozas, Madrid, Spain). The GC injection port was set at 270 °C in splitless mode (purge time 0.5 min). The GC was equipped with a J&W 5% phenylmethylsyloxane capillary column, 30 m × 0.25 mm. i.d., 0,50 μm film thickness (purchased by Agilent Technologies, Las Rozas, Madrid, Spain). The oven temperature was held at 100 °C for 1 min, then at 42 °C min^{-1} to 200 °C, then at 15 °C min^{-1} to 280 °C and held 12 min. Helium was used as carrier gas at a flow of 1 ml min^{-1}. The mass detector operated in electron ionization at 70 eV. Initially, a mixture of standards of all the compounds was analysed in full scan mode (mass range 50–550 amu). Quantifier and qualifier ions used for each analyte were selected on the basis of their abundance and mass-to-charge ratio (*m/z*). Owing to their reproducibility and lack of interferences, high mass ions were selected whenever possible. Upon selection of ions, the mass analyzer was operated in selected ion monitoring (SIM) acquisition mode. All diagnostic ions and retention times are listed in Table 1.

Table 1. Retention times and diagnostic ions. Ions in bold are used for quantitation

Compound	Retention time	Ions (*m/z*)
Norfentanyl-PFP	12.8	**150**, 322, 229
Fentanyl	17.7	**245**, 146, 189
Sulfentanyl	18.5	**289**, 140, 187
Alfentanil	21.1	**289**, 222, 268
Fentanyl-D5	17.7	**250**, 151, 194

Method Validation

Limits of detection, lower limit of quantitation and specificity

The limit of detection (LOD) value was considered the concentration value giving a S/N > 3 for at least three diagnostic ions for each substance, while the lower limits of quantitation (LLOQ) was the lowest level of the calibration curve if the following conditions were met: (1) the analyte response at LLOQ was at least 5 times the response compared to a blank response; and (2) the analyte peak was identifiable, discrete and reproducible with a precision of 20% and an accuracy of 80–120%.

Specificity was studied analyzing 10 negative urine samples. The method was also applied to real samples from subjects taking common over-the-counter medications such as diclofenac, ibuprofen, salicilates, tramadol, flurbiprofen pseudoephedrine or metoclopramide.

Linearity

The linearity of the method for each compound was studied in the range 0.5–50 ng ml^{-1}, performing three extractions and analyses for each level. Calibration curves were built by linear regression of the area ratio of each substance with the internal standard (IS) vs the concentration of each analyte. Curves with a quadratic regression coefficient (R^2) higher than 0.99 were considered to be satisfactory.

Precision and accuracy (bias)

The repeatability (intra-assay precision) of the method was studied on five replicate analyses at three levels: 0.5, 10 and 50 ng ml^{-1}. Inter-day precision was assessed by analyzing three aliquots for each of the above concentrations on five different days. The analytical accuracy for each analyte was expressed as the percentage deviation of mean calculated value from the theoretical sample concentration (mean relative error, %E).

Recovery

Extraction efficiency was evaluated at two levels, 0.5 and 50 ng ml^{-1}, by analyzing five replicate extracted samples versus samples spiked with the standards after the extraction step.

RESULTS AND DISCUSSION

The method developed allowed the determination of the analytes in a relatively short time and with a good sensitivity. The derivatization step was necessary in order to improve the sensitivity for norfentanyl, which showed a poor chromatographic behavior and a lack of sensitivity if injected underivatized. The structures and postulated fragmentations of the compounds studied are shown in Fig. 1.

All the analytes could be detected with an LOD of 0.08 ng ml^{-1} (0.04 ng ml^{-1} for alfentanyl) and an LLOQ of 0.5 ng ml^{-1}. The method was linear in the range 0.5–50 ng ml^{-1} with quadratic regression coefficients ranging from 0.9903 to 0.9926 (Tables 2 and 3). The repeatability of concentrations and accuracy were acceptable for all the substances (coefficients of variation, CV, of concentration values and mean analytical error were lower than

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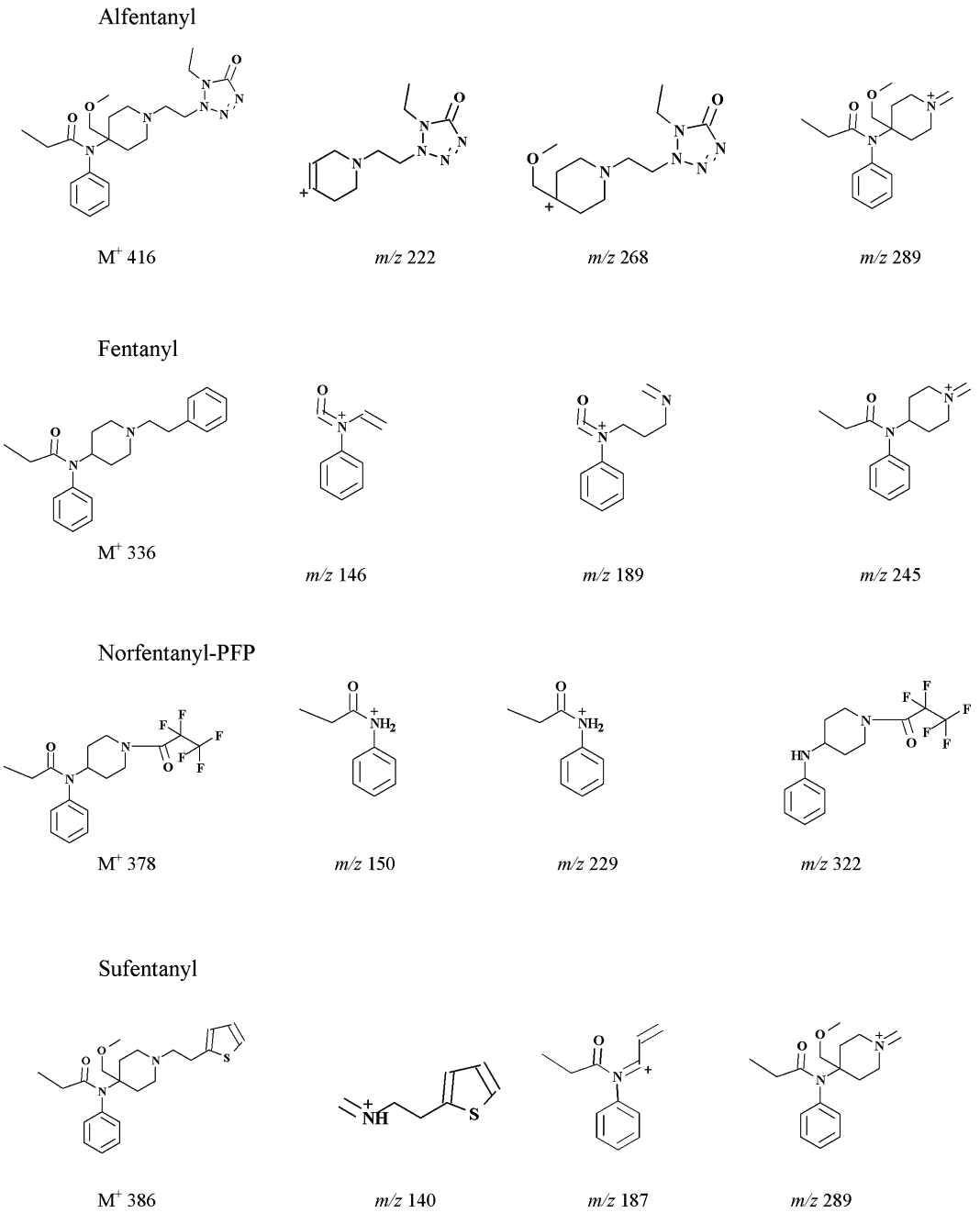

Figure 1. Structure and postulated fragmentations of alfentanyl, fentanyl, norfentanyl-PFP and sufentanyl.

15% for all the compounds studied, both for intra- and inter-day experiments). Results from the validation study are summarised in Tables 2 and 3. The analyses performed on 10 negative samples did not show significant interferences at the retention times of the analytes. This confirms that the method possesses adequate selectivity.

Figure 2 shows a chromatogram of a negative urine, while Fig. 3 shows a chromatogram from a blank urine spiked with all the substances studied at a concentration of 10 ng ml^{-1}. As can be seen, all the analytes are well separated, and can be identified by their characteristic fragment ions and retention times.

The method was applied to four real urine samples from subjects submitted to general anesthesia by a department of general surgery. Fentanyl was identified in all the subjects at concentrations ranging from 0.8 to 4.0 ng ml^{-1}; norfentanyl could be quantified only in the sample with the higher fentanyl concentration, at 1.6 ng ml^{-1}. In the other cases the estimated norfentanyl concentrations were below LLOQ. Figure 4 shows a chromatogram of a urine sample from a subject administered a therapeutic dose of fentanyl (100 µg).

The method was also applied to a case of suicide by ingestion of fentanyl patches. In that case the concentrations of fentanyl and norfentanyl encountered in urine, calculated after dilution 1 : 10 of the sample with a blank urine, were respectively 183 and 76 ng ml^{-1}.

Table 2. Validation results: intra- and inter- day precision and accuracy

	CV% intra-day			CV% inter-day			%E intra-day			%E, inter-day		
Compound	0.5 ng ml^{-1}	10 ng ml^{-1}	50 ng ml^{-1}	0.5 ng ml^{-1}	10 ng ml^{-1}	50 ng ml^{-1}	0.5 ng ml^{-1}	10 ng ml^{-1}	50 ng ml^{-1}	0.5 ng ml^{-1}	10 ng ml^{-1}	50 ng ml^{-1}
Norfentanyl	6.53	1.92	10.55	18.29	14.43	9.69	7.78	0.28	5.12	3.99	1.41	1.32
Fentanyl	3.23	7.29	6.75	14.22	9.82	5.62	14.80	13.76	2.92	14.72	13.06	2.52
Sufentanyl	8.33	9.67	9.85	4.38	11.09	8.59	13.02	12.72	13.73	8.82	13.35	0.057
Alfentanyl	14.52	4.42	8.85	9.29	12.34	9.18	0.14	9.61	10.10	6.48	6.76	0.04

Table 3. Validation results: linearity and recoveries

Compound	Slope	Intercept	Slope standard error	R^2	Recovery 0.5 ng ml^{-1}	CV%	Recovery 50 ng ml^{-1}	CV%
Norfentanyl	0.052	0.072	0.0026	0.9903	87%	10.6	89%	9.9
Fentanyl	0.047	–0.019	0.0017	0.9914	85%	11.6	96%	11.3
Sulfentanil	0.105	–0.031	0.0032	0.9926	92%	12.3	95%	11.5
Alfentanil	0.444	0.011	0.014	0.9925	86%	9.5	89%	8.6

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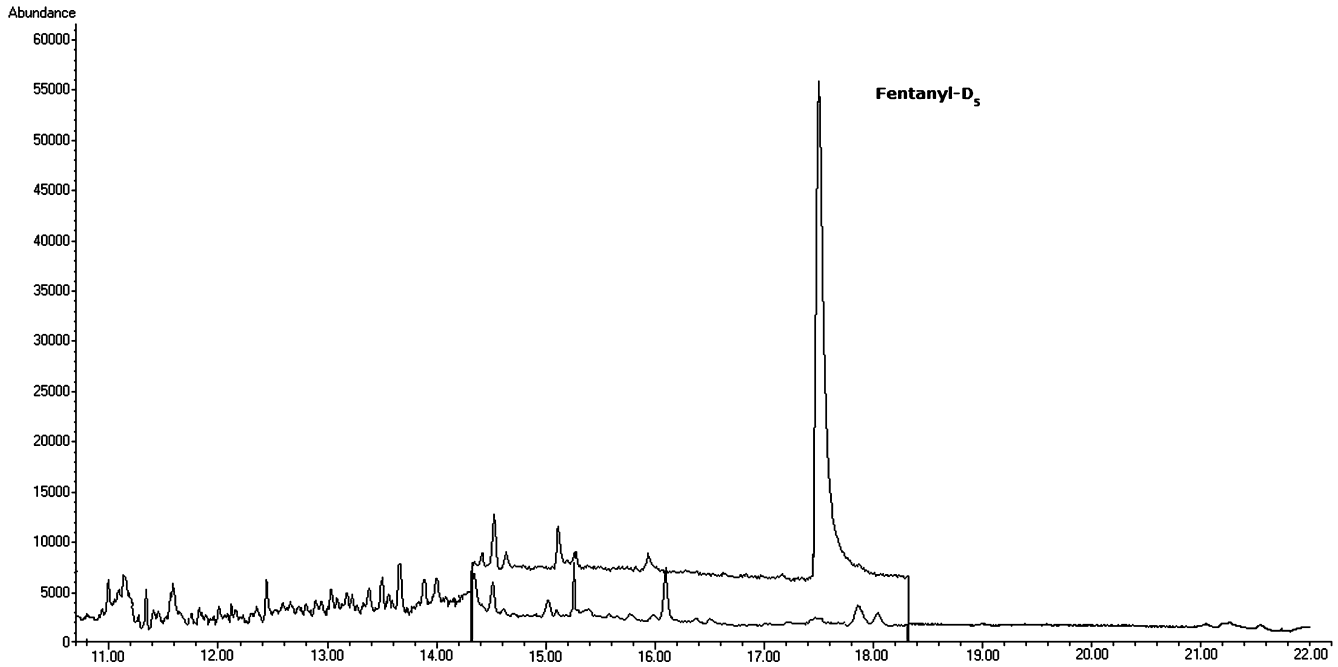

Figure 2. Extracted ions chromatogram of a blank urine sample.

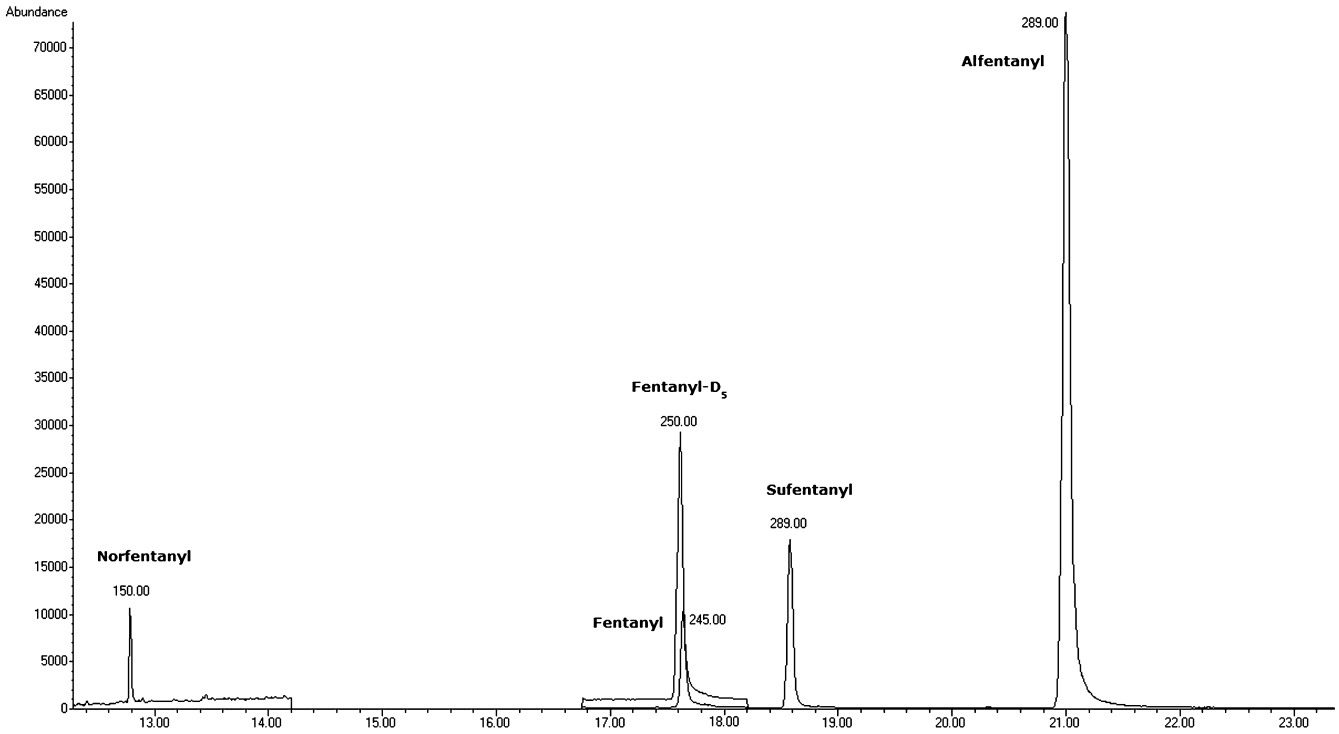

Figure 3. Extracted ions chromatogram of a blank urine sample spiked with fentanyl (*m/z* 245), norfentanyl (*m/z* 150), alfentanyl (*m/z* 289) and sufentanyl (*m/z* 289) at a concentration of 10 ng ml^{-1}.

The method, applied to real samples, both in therapeutic and in overdose concentrations, was demonstrated to be suitable for its application in forensic toxicology. The LOD at 80 pg ml^{-1} is appropriate for the determination of fentanyl analogs and metabolites in urine also at low therapeutic concentrations, in addition to overdose concentrations. The sensitivities are also suitable for the confirmation of fentanyl and analogs in antidoping controls, where a minimum performance level of 10 ng ml^{-1} is required (World Antidoping Agency, 2010b).

In conclusion, the proposed fully validated method allows the sensitive determination of fentanyl and its main metabolite and analogs by GC/MS, after a simple sample pre-treatment. The

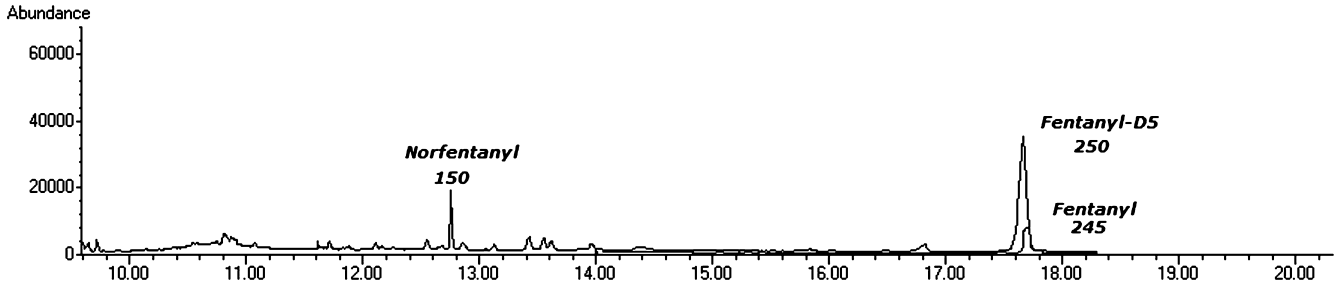

Figure 4. Extracted ions chromatogram of a real urine sample after a therapeutic administration of fentanyl.

LLODs obtained, lower than those generally reported in other studies, are fully satisfactory for its application to forensic toxicology, including anti-doping analyses, as demonstrated also by its application to real samples from surgery patients and postmortem samples.

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