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Gas chromatographic–mass spectrometric quantitation of dextropropoxyphene and norpropoxyphene in hair and whole blood after automated on-line solid-phase extraction Application in twelve fatalities

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Abstract

After conversion of norpropoxyphene (NP) to its corresponding amide, dextropropoxyphene (DP) and NP are extracted from 1 ml of blood or 50 mg of powdered hair, on C_{18} cartridges and eluted using methanol containing 0.5% acetic acid. Automated extraction is conducted on-line with automated device, starting from buffered and centrifuged sample. After extraction, the dried residue is reconstituted with 40 μl of methanol, and then injected in a gas chromatograph at 250°C. Quantitation is carried out by gas chromatography–mass spectrometry in the selected-ion monitoring mode, lidocaine being the internal standard. The method gave relative standard deviations lower than 6.2% in whole blood, and 6.0% in hair for the entire range of calibration from 0.5 to 10 μg/ml in blood and from 1 to 20 ng/mg in hair of both compounds. Limits of detection in blood and hair for DP are, respectively, 0.07 μg/ml and 0.05 ng/mg, whereas the respective limits of detection in whole blood and hair for NP are 0.09 μg/ml and 0.04 ng/mg. The present method was used for one year in our laboratory. Postmortem concentrations of DP in blood ranged from 1.6 to 44.0 μg/ml (mean=9.8 μg/ml, n=12) and are comparable to those found in the literature. Out of 30 hair samples from people who died from heroin overdose, 13 were positive both for DP and NP with concentrations ranging from 0.2 to 27.4 ng/mg (mean 8.7 ng/mg) for DP and 0.3 to 68.9 ng/mg (mean 24.1 ng/mg) for NP.

Keywords: Dextropropoxyphene; Norpropoxyphene

1. Introduction

Dextropropoxyphene (DP) is a weak opioid, an analgesic, taken by mouth for the control of mild to moderate pain [1,2]. Generally combined with paracetamol or aspirin, the drug can also be dispensed alone as pure hydrochloride. Since its introduction in the '60s, DP has been prescribed extensively, and there has been a significant increase in the number of reported fatal poisonings by the drug. Christensen [3,4] examined postmortem concentrations of the drug and its main metabolite (norpropoxyphene= NP) in blood and tissues samples, finding blood levels from 0.7 to 12.0 μg/ml in 53 fatal cases. Quantitation was performed by gas chromatography (GC) using flame ionization detection and a tedious multi-step liquid–liquid extraction. Christensen and others, furthermore, documented the use of thin-layer

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chromatography as a semi-quantitative method [5]. Norheim [6] used gas chromatography–mass spectrometry (GC–MS) in autopsy samples, and immunoassay for urine [7]. Several published methods for the simultaneous quantitation of DP and its metabolite employ a conversion of NP to its corresponding amide (NPA) before final extraction [7–9]. Norheim documented the quantitation of hydrolyzed esters of DP and NP [6].

Although liquid–liquid extraction is the most common procedure to extract the analytes, King and King [7] developed a very efficient solid-phase extraction (SPE) for urine analysis. However, this procedure used cartridges packed with only 15 mg of sorbent which is not well suited for a complex matrices such as putrefied whole blood or tissue extracts.

In our forensic laboratory, DP is a relatively frequent finding. Over a period of one year, 12 fatalities were due to DP (just behind meprobamate: 19 fatalities) out of 124 caused by pharmaceuticals [10]. DP is an opiate agonist, thus addicts can switch to DP, codeine or other antitussives as these drugs are easier to find and at lower cost than heroin. Recently these drugs have been also quantified in hair from overdose cases by an high-performance liquid chromatography (HPLC) technique [11]. However, limits of detection were about 1.0 ng/mg hair for NP and higher than 1.5 ng/mg hair for NP.

In light of the problems encountered with previous quantitation methods and the frequency of DP intoxications, we have developed a very simple method for automated SPE of DP and NP in whole blood and hair coupled with a selective and sensitive GC–MS assay in the selected-ion monitoring (SIM) mode.

2. Experimental

2.1. Instrumentation

The instrumentation used was composed of a 5890 Series II Plus gas chromatograph, a Model 7673 automatic injector and a Model 5972 mass selective detector (Hewlett-Packard, Les Ulis, France). The SPE unit was a Model 7686 Prepstation Hewlett-Packard. The analytical column was a CP SIL 8 CB, 25 m×0.25 mm I.D. (0.25 μm film thickness) from Chrompack (Les Ulis, France). Helium was used as the carrier gas at a flow-rate of 1.3 ml/min in the constant flow mode (92 KPa at 130°C).

Analytical conditions for DP and NP were as follows: detector temperature, 280°C; splitless injection (2 μl) at 250°C; initial oven temperature was 130°C for 1 min and then increased to 210°C at 25°C/min and held for 6.8 min; finally increased to 280°C at 25°C/min and held for 2.2 min. The analytical run time was 16 min. Retention times (t_Rs) were 5.7, 9.5 and 14.0 min for lidocaine (internal standard=I.S.), DP and NPA, respectively. Relative t_R (RRT) of analytes to I.S. was 1.67 for DP and 2.46 for NPA. Collected ions were m/z 86, 234 for lidocaine (4 to 8 min), m/z 58, 91, 105 for DP (8 to 12 min) and m/z 91, 105, 234 for NPA (12 to 16 min). The dwell time per ion was set at 175 ms.

For SPE, we used C_{18} cartridges, 100 mg from Hewlett-Packard (Part No. G1204-62092). Two high recovery vials (part number 5182-3454) were used for both the buffered samples (blood or hair) and the acidic extract obtained after elution from SPE cartridges. The Prepstation was supplied with seven fluids: water, methanol 10% in water, methanol, methanol acidified with 0.5% acetic acid (eluting solvent), bicarbonate buffer, 2% trifluoracetic acid (TFA) in water and air. The first five fluids were used for the SPE procedure, while 2% TFA was used to rinse the whole system between extractions. Air was employed both as a drying gas for the SPE cartridges and during the heated evaporation of the eluate. The distribution syringe of the Prepstation had a capacity of 2.5 ml.

The hair pulverizer was a Model 4200-EI from KLECO (Visalia, CA, USA).

2.2. Injection conditions

Thermal degradation of DP described by Millard et al. [12] gives rise, together with DP, to two diphenylbutene isomers, for injection temperature between 210 and 250°C with a corresponding maximum DP loss of 7%. Above 260°C, a second degradation product is formed by the loss of propionic acid. Therefore, to obtain a satisfactory flash volatilization of NPA and to minimize degradation of DP, a temperature of 250°C was chosen, as already reported by Amalfitano et al. [13].

Concerning NP, this molecule exhibits poor gas chromatographic behavior, as already mentioned by numerous authors. Without transformation, the drug elutes, giving three peaks at different t_Rs with the same mass spectra. However, if NP is brought to pH 11 or above, it undergoes an intramolecular acyl shift leading to a stable molecule: norpropoxyphene amide. The propionic group is transferred to the secondary amine of NP, this results in a compound which can be easily chromatographed in a unique and well resolved peak. Linearity of the transformation of NP to NPA and the stability of DP and NPA under strong alkaline conditions has already been demonstrated [13]. Since NPA generates high-mass ions (main ions at m/z=234, 105, 100, 91 and 44) limit of detection of NP can be dramatically improved as compared to HPLC–UV techniques.

2.3. Reagents

Methanol, glacial acetic acid, 0.1 *M* hydrochloric acid, 1 *M* sodium hydroxide, trifluoracetic acid and sodium bicarbonate ($NaHCO_3$) were from Carlo Erba (Milan, Italy). Lidocaine was generously offered by Laboratoires Sepval (Laval, France), DP and NP were supplied in 1-ml vials (100 μg/ml in methanol) by Promochem (Molsheim, France).

2.4. Sample collection and decontamination

Hair is best collected from the back of the head – an area called the vertex posterior – which exhibits less variability in hair growth rate than other areas. The sample size collected was at least 150–200 mg.

Environmental contamination of hair by drugs of abuse has been extensively studied and reviewed by a number of authors. However, no universal procedure seems to adequately eliminate all types of contamination [14–17]. Regarding hair analysis of DP, the risk of contamination seems low since the drug is normally taken in tablets. In reality while passive exposure to smoke or powders dispersed in the environment is possible for cocaine or heroin, these factors appear to be insignificant for pharmaceuticals such as DP. However, in the absence of more information, we performed the same decontamination protocol we observe for opiates and cocaine [14]. It includes two washes for 3 min each with dichloromethane followed by two 3-min washes with a phosphate buffer (0.01 *M*, pH 5.6). The dichloromethane washes offer the advantage of removing fat from the hair before acidic solubilisation.

The samples were cut into 2 cm segments which were pulverized separately in the hair pulverizer.

2.5. Calibration and reagent solution

A stock solution containing 50 μg/ml of lidocaine free base was prepared in methanol and stored in a screw cap vial at −30°C for a maximum of three months. An equivalent stock solution of DP and NP was realized at a concentration of 10 μg/ml and stored under the same conditions. The solution for conditioning the SPE cartridges was prepared by dissolving 16.8 g of sodium bicarbonate in 1 l of 10% methanol in water (0.2 *M*), pH 8.6 (pH adjustment was unnecessary).

Drug-free biological samples were collected from drug-free volunteers. DP–NP standard solution was added to negative hair producing concentrations of 1, 2, 10 and 20 ng/mg. Spiked blood was prepared at concentrations of 0.5, 1, 2 and 10 μg/ml. Drug-free samples were collected from screened volunteers. Biological matrices used as blanks were pooled, extracted and analyzed by the present method: no peak was obtained which corresponded to the compounds of interest.

2.6. Extraction procedure

2.6.1. Whole blood

Fifty μl 1 *M* sodium hydroxide and 100 μl of I.S. stock solution (50 μg/ml) were successively added to 1 ml of whole blood and vortex mixed for 15 s. Rearrangement by base catalysis of NP to NPA was obtained at room temperature in 5–10 min. Two ml of bicarbonate buffer were added, then the prepared sample was vortex mixed for 15 s and centrifuged at 7500 *g* for 6 min. 1.5 ml of the supernatant was transferred to a high recovery vial and positioned on the HP 7673 tray. Near this vial, a C_{18} cartridge was placed, close to another sealed high recovery vial ready to contain the final injection extract.

The dispense speed of the washing solvents was set at 1 ml/min and that of the eluting solvent was

set at 0.7 ml/min. 1.5 ml of the sample was aspirated and loaded on the sample loop of the automated device. C_{18} cartridges were successively conditioned with 2 ml methanol and 1 ml bicarbonate buffer. Loaded sample was then applied to the cartridge which was washed twice with 0.5 ml water and once with 0.5 ml 10% methanol. The column was then allowed to dry by passing air through for 10 min. Entire system flow path was rinsed with 5 ml acidified methanol before performing the elution of the column with 600 μl of the eluting solvent and then with 700 μl of air (used as solvent plug). Evaporation was realized at 70°C at three different needle heights as a function of time: 18, 15.5 and 13 mm during 7, 3 and 2 min, respectively. Reconstitution was realized with 40 μl of methanol and 2 μl were injected onto the GC system. The SPE device was rinsed with 10 ml 2% TFA and 10 ml water before performing another extraction. The detailed Prepstation method is available on floppy disk by request.

2.6.2. Hair

Fifty mg of powdered fortified hair or unknown samples were added to a conical screw cap vial in which 10 μl of I.S. methanolic solution (50 μg/ml) and 700 μl of 0.1 *M* hydrochloric acid were added. After incubation at 56°C for 12 h, the acidic medium was alkalized with 120 μl of 1 *M* sodium hydroxide and left at room temperature for 5 min, then buffered with 1 ml of bicarbonate buffer (pH 8.6). After centrifugation at 2500 *g* for 5 min, 1.5 ml of the supernatant was transferred into a high recovery vial and processed as blood.

3. Results

3.1. Precision and recovery

The relative standard deviations (R.S.D.s) of the within-day precision ($n=8$) were always less than 3.2% in whole blood and less than 2.7% in hair for DP. For NPA, they were less than 3.4% and 3.5% in the same matrices, respectively. R.S.D.s for DP in the between-day precision study were 5.1, 4.9, 3.0 and 1.2% in whole blood at 0.5, 1, 5 and 10 μg/ml, respectively. In hair they were equal to 6.0, 6.3, 4.0 and 1.9% at the following concentrations: 1, 2, 10 and 20 ng/mg. For the same concentrations R.S.D.s of NPA were 6.2, 4.2, 3.0 and 2.9% in whole blood and 5.9, 4.0, 3.2 and 3.4% in hair, respectively.

The overall DP recovery in whole blood was 92.5% at 1 μg/ml and 90.6% at 10 μg/ml ($n=8$). Overall recovery in hair was equal to 86.5 and 87.1% at 2 and 10 ng/mg, respectively ($n=10$). NPA recovery was 93.5% in blood at 1 μg/ml ($n=8$) and 85.2% in hair at 2 ng/mg ($n=10$).

3.2. Linearity

Analysis of variance (ANOVA) is a powerful and very general method which separates the contributions to the overall variation in a set of experimental data and tests their significance. the sources of variations are each characterized by a sum of square (SS), i.e., the sum of a number of squared terms representing the variation in question, a number of degrees of freedom (DF), and a mean square, which is the former divided by the latter and which can be used to test the significance of the variation contribution by means of the *F*-test. In analytical calibration experiments, only variation in the *Y*-direction is considered. This variation is expressed as the sum of the squares of the distances of each calibration point from the mean *Y* value. This is the total SS of Y_{mean}: S_T. There are two contributions to this over-all variation. One is the SS due to the regression: S_1, and the second source of variation is the SS about regression, i.e., residual: S_0. The residual variation can be divided itself into two contributions: lack of fit and pure analytical error. F_{cal} (whole blood)= 3256.2, F_{cal} (hair)=4156.8>>F_{theor}; F_{theor} is for (1, $n-2$) DF=5.32, so the source of variation is well described by the regression, F'_{cal} (whole blood)= 2.02, F'_{cal} (hair)=3.12<F'_{theor}; F'_{theor} is for [$(n-2)-n/2$, $n/2$] DF=5.41, so the model (linear regression) can be considered as correct [18–20]. The same assertion was verified for NP. Equations were: DP, whole blood: $Y=0.9921X-0.0026$ [(amount ratio (DP/I.S.)/response ratio (DP/I.S.)]; DP, hair: $Y=2.0702X-0.0098$ [(amount ratio (DP/I.S.)/response ratio (DP/I.S.)]; NP, whole blood: $Y=0.9987X-0.0007$ [(amount ratio (NP/I.S.)/response ratio (NP/I.S.)] and NP, hair: $Y=2.0434X-0.0032$ [(amount ratio (NP/I.S.)/response ratio (NP/I.S.)].

3.3. Accuracy

In a first step, a blood standard of known concentration is prepared and measured n times. From the values of the peak areas, we can calculate the mean m, and the standard deviation S.D. which we consider as being the true values.

In a second step, from the equation of the linear regression: $Y=aX+b$, we can calculate the measured peak area corresponding to this point: Y_t. Then we test the accuracy by the mean of a t-statistic; by comparing a mean to a point as follows: $t_{observed}=(m-Y_t)/(S.D./n^{1/2})$. If $t_{observed}<t_{table}$, the null hypothesis is accepted; in other words, the measured value and the true value are not different. t_{table} is given with $n-1$ DF.

Using this procedure, only internal errors were tested. Since the method for standard preparation was always the same, we cannot estimate the lack of accuracy due to the external errors (incorrect mass or volumes of standards or samples, or inaccurate dilution of primary standards).

Under these conditions, $t_{observed}$ was always inferior to $t_{table}=2.26$ ($\alpha=5\%$, $n=10$).

3.4. Limits of detection (LODs)

The LOD is given by the mathematical formula: $LOD=m_{blank}+6s_{blank}$, where m_{blank} is the mean of the blanks and s_{blank} corresponds to the S.D. of the blanks ($n=30$) [18–21].

Under these conditions, LODs of DP were: 0.07 μg/ml in blood and 0.05 ng/mg in hair; LODs of NPA were: 0.09 μg/ml in blood and 0.04 ng/mg in hair.

4. Forensic applications

4.1. Whole blood concentrations in 12 fatalities

The concentrations of DP measured during one year of forensic activity ranged from 1.6 to 44.0 μg/ml (mean=9.8 μg/ml) in 12 fatalities that the coroner attributed as being essentially due to a DP overdose (Table 1 and Fig. 1). DP was, thus, the main cause of death in 12 out of 124 fatalities attributed to therapeutic drug overdose. DP was second only to meprobamate (19 fatal cases) [10]. This compound was also found at therapeutic concentrations in 16 out of 124 cases of therapeutic drug overdose and in 81 heroin overdose fatalities out of 202 cases. Thus, its total frequency can be considered very significant.

Norheim [6] reported six fatal intoxications with DP blood concentration ranging from 1 to 11 μg/ml (mean=4.3 μg/ml). Worm [5] published nine fatalities; ranging from 0.4 to 23.0 μg/ml (mean=4.8 μg/ml). Christensen [3] published 29 fatalities with blood concentrations ranging from 0.7 to 15.0 μg/ml (mean=4.6 μg/ml). Thus, our results appear consistent with the literature.

4.2. Hair concentrations in 13 cases

Thirty hair samples, obtained from drug addicts deceased from heroin overdose, were subjected to DP and NP analysis. Thirteen were positive both for DP and NP. These findings are comparable to our previous data on blood, i.e., 81 positive out of 202 heroin overdoses. The measured concentrations in hair were in the range 0.24 to 27.41 ng/mg (mean 8.7 ng/mg) and 0.36 to 68.94 (mean 24.1 ng/mg) for DP and NP, respectively. The mean ratio, metabolite/parent drug, was 2.77 (extreme figures 0.36 to 6.24).

Mersch et al. [11] reported the concentrations in hair of 11 people tested positive for DP. In 21 segments (out of 24 analyzed) concentrations ranged between 1.2 to 26.4 ng/mg for DP and 2.9 to 71.0 ng/mg for NP. Ratios of NP/DP were between 0.28 and 4.76 (mean 1.09).

The mechanism generally proposed for the endogenous pathway of molecules into growing hair is passive diffusion from the blood supply at the base of the hair follicle. When a drug wants to permeate a cell, it must traverse the plasma membrane. Thus, we must consider the mechanisms by which drugs cross membranes and the physicochemical properties that influence this transfer. Most drugs are weak bases or acids that are present in biological fluids both in their ionized and non-ionized forms. Plasma membrane exhibited a low permeability to polar molecules, thus ionized compounds are rarely able to penetrate the lipid cell membrane. Only the free, non-ionized molecules that are sufficiently lipid soluble, can

Table 1
Postmortem blood concentrations of DP, NP and other associated drugs

DP (μg/ml)	NP (μg/ml)	Alcohol (g/l)	Associated drugs (μg/ml)
1.6	2.1	2.4	–
1.9	3.8	0.8	Paracetamol=40.2
2.0	1.6	–	Codeine=0.042 Morphine=0.105
2.9	1.4	1.1	Flunitrazepam=0.065
3.9	1.7	–	–
4.2	4.3	2.3	Codeine=0.016 Fluoxetine=0.58 Meprobamate=20.2 Morphine=0.046 Nordazepam=0.65 Paracetamol=22.3
4.3	0.7	–	Codeine=0.065 Morphine=0.405 Nordazepam=3.0 Oxazepam=0.9
8.9	4.7	0.9	–
12.5	5.4	–	Flunitrazepam=0.142 Nordazepam=1.6 Oxazepam=0.4
14.1	3.2	–	Zopiclone=2.42
16.8	13.8	–	–
44.0	10.7	–	Paracetamol=512.0

– : Not detected.

diffuse across the cell membrane. Once inside the cell, the uncharged drug will dissociate depending upon its pK_a value. The pH dependence of dissociation and lipid solubility of a substance may result in the unequal distribution of the drug in various compartments with different pH values. This favor the accumulation of undissociated basic drugs in the compartment with the lower pH. The isoelectric pH of hair being close to 6, it clearly indicates the acidic nature of the hair [22], and thus the good incorporation of basic drugs.

Another very important point, is represented by the drug interaction with melanin. In contrast to matrix cells, the melanocytes are a highly specialized cell population. The intracellular pH for melanocytes has been estimated in the range 3–5 [23]. Accumulation of basic substances is favored in the cytosol. Many investigators have demonstrated the affinity of natural and synthetic melanins to various drugs [24]. It is generally accepted that the ability of melanin-containing tissue to accumulate and retain drugs is remarkable [25]. Being entrapped during melanin granule formation, a permanent concentration gradient will occur for drugs with a high melanin affinity and the influx into a melanocyte will be higher than into a matrix cell. According to the melanin and acidic protein concentrations contained in white or black hair, it is natural to consider that black hair can concentrated basic drugs more efficiently while white hair could incorporate acidic drugs slightly more effectively. In all cases the basic drugs have a better incorporating ratio than the acidic ones. Nevertheless, the low incorporating ratio of acidic drugs is well balanced by a high blood concentration which can be a thousand times higher than the one of a basic drug.

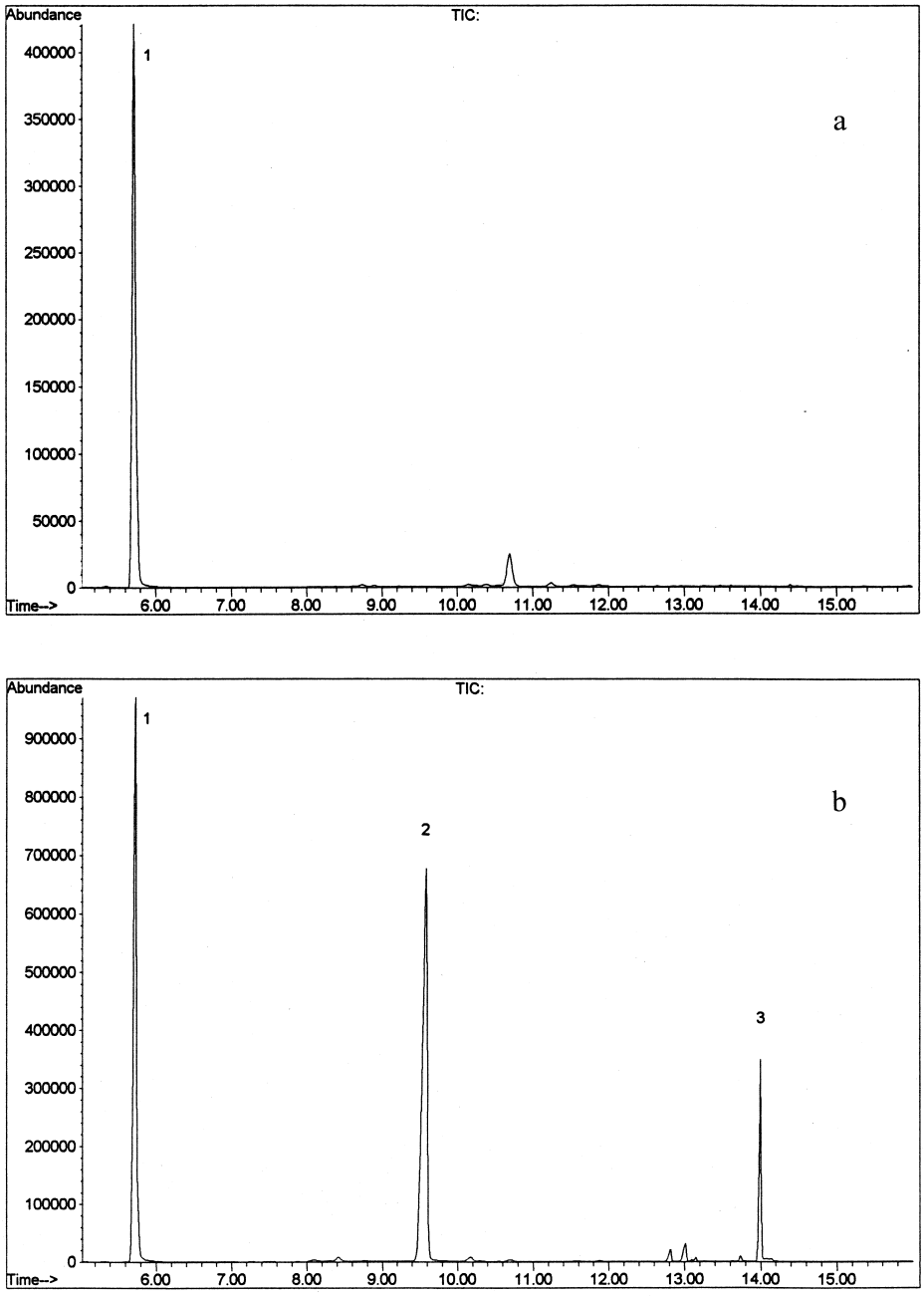

Fig. 1. Total ion current chromatogram of an extract of 1 ml of (a) blank whole blood. Peak: 1=I.S. and (b) whole blood of the deceased No. 5. Peaks: 1=I.S.; 2=dextropropoxyphene (3.92 μg/ml); 3=norpropoxyphene amide (1.73 μg/ml).

Slightly less hydrophobic than its parent drug, NP has a half life time (36 h) around three times higher than that of DP (12 h) [26]. Therefore due to favorable pharmacokinetic properties and according to our previous findings for thiopental and pentobarbital [27], it seems that DP main metabolites

can also strongly accumulates into hair leading to a ratio metabolite/parent drug higher than 1.

Due to the lack of information on DP dosage, it was not possible to evaluate a potential correlation between concentrations in hair and daily dose. The following case represents the only known daily consumption we have seen in our laboratory.

4.3. Hair analysis of a drug user: case report

The abuser was a 23-year-old male with a heroin abuse history dating back three years. Police arrested him for dealing heroin and firearms. Interrogated about his drug use, he said he had stopped consuming heroin nine months before and started daily consumption of two 65 mg tablets of DP. Because his hair had been recently shaved, pubic hairs were taken for analysis. Performed in our laboratory, results of codeine, morphine, 6-acetylmorphine were negative. In a second step, analysis of DP in hairs showed a consumption of this drug. Fig. 2 displays chromatograms from an extract of 50 mg of powdered pubic hairs. DP was measured at a concentration of 24.84 ng/mg, whereas that of NP was 134.90 ng/mg (see Fig. 1).

5. Conclusions

The present method highly sensitive, produces a cleaner extract and a clean chromatographic profile which can enable the analyst to solve difficult cases sometimes encountered in forensic medicine – in particular, complex or putrefied matrices. Moreover, automation of the entire procedure gives a very reproducible method, reducing work and increasing productivity. Investigation of unconventional samples such as hair is an interesting prospective tool in documenting and studying personal drug history.

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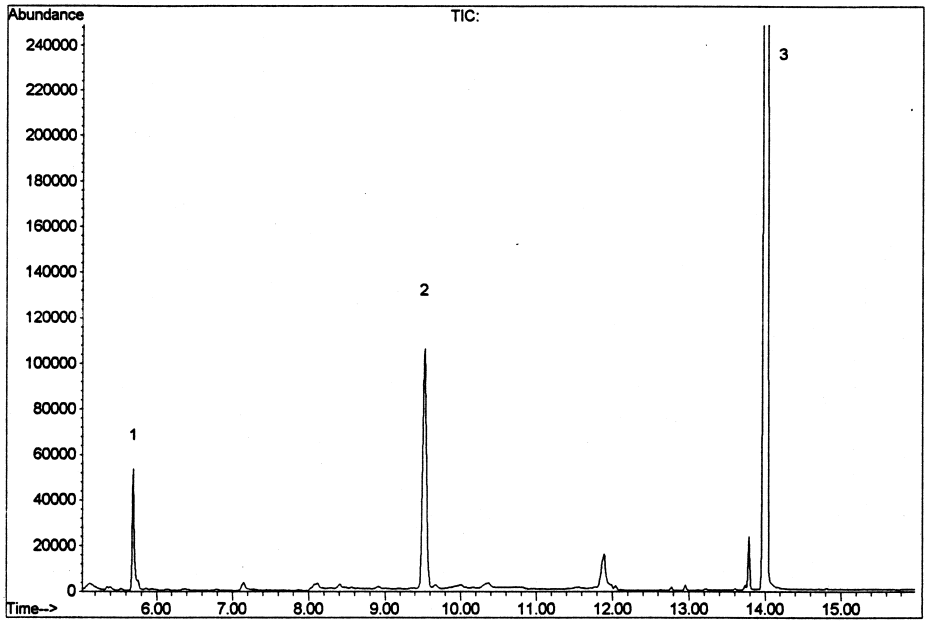

Fig. 2. Total ion current chromatogram of an extract of 50 mg of pubic hair taken from a drug user of 130 mg daily of dextropropoxyphene by mouth for nine months. Peaks: 1=I.S.; 2=dextropropoxyphene (24.84 ng/mg); 3=norpropoxyphene amide (134.90 ng/mg).

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