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Screening and identification of drugs in human hair by high-performance liquid chromatography–photodiode-array UV detection and gas chromatography–mass spectrometry after solid-phase extraction
A powerful tool in forensic medicine

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Abstract

A method is described to screen for a wide range of pharmaceuticals in human hair. 75 mg of powdered hair are incubated (12 h at +56°C) in 2 ml of distilled water (acidic compounds) or 0.1 *M* hydrochloric acid (neutral and basic compounds). A twin solid-phase extraction on C_{18} cartridges is used for the sample clean-up procedure. Acidic drugs are fixed at pH 2 and eluted with 1% ammoniacal methanol while neutral and basic drugs are retained on the column at pH 8.6 and eluted with methanol containing 0.5% acetic acid. The internal standard (I.S.) for the acidic extraction was bupivacaine while the I.S. for the basic extraction was prazepam. The separation of the drugs was performed using both the liquid and the gas chromatographic techniques whereas identification was achieved using photodiode array and mass spectrometric detection, respectively. The liquid chromatographic system gives an elution of the drugs following a multi step gradient from a Symmetry C_8 (Waters) 5 μm column (250×4.6 mm I.D.) at +30°C with acetonitrile–phosphate buffer (pH 3.8). Identification is achieved using the reference data (retention times and spectra) of 675 pharmaceuticals, toxicants and drugs of abuse stored in a personal library. The present method has been applied during 6 months in our laboratory. By establishing a victim's drug use history, it is a very powerful tool in forensic medicine. We illustrate the method with some real cases of police crime investigation.

Keywords: Hair; Forensic analysis; Toxicological screening; Extraction methods; Drugs

1. Introduction

The number of papers relating to drug detection in hair has increased exponentially during the last 15 years. This interesting alternative matrix has been used for poison detection, (mainly arsenic), since 1857 by Casper [1] in his very famous Praktisches Handbuch der Gerichtlichen Medizin. About one hundred years later, in 1954, the first organic drug (barbiturate) was detected in guinea-pig hair [2]. In 1979 Baumgartner et al. [3] published a radioimmunological method for the detection of opiates in the hair of drug addicts and introduced the idea of tracking drug use for the first time. From this date, great interest has been shown in this matrix in forensic cases where the detection of illegal drug use, has been determined by dating drug consumption, and has been illustrated in numerous celebrated forensenic cases throughout the world. Furthermore, the press has made this method popular. On the other

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hand, due to the improvement of analytical procedures, the number of molecules identified has also increased and continues to progress. After identification of morphine [4] and 6-acetylmorphine [5], a marker of heroin intake, papers dealing with cocaine and its metabolites were extensively published [6–8], together with the detection of special metabolites such as cocaethylene (cocaine and alcohol consumption) [9] or, as anhydroecgonine methylester (marker of crack consumption) [10]. Detection of methadone [11], amphetamines [12–15], cannabinoides [16–18] and phencyclidine [19] were also reported.

Concurrently the identification of numerous pharmaceuticals has been achieved. In fact, pharmaceutical analysis of hair can be a useful tool for drug monitoring, drug compliance, and of course, can present great advantages in forensic medicine in the establishment of a personal drug history or when classical matrices are not available (putrefied bodies). Kintz et al. [20] have reported a gas chromatography–mass spectrometric (GC–MS) method for the quantification of meprobamate in hair and have shown the correlation between drug levels in hair and in serum. The same observation was made between drug levels and 23 clinical observations for phenobarbital by Goullé et al. [21]. A significant relationship was also found between the hair concentration of haloperidol and daily intake of this antipsychotic in 40 patients [22]. Haloperidol was detected in hair samples by isocratic high-performance liquid chromatography (HPLC) [23] with ultraviolet (UV) detection. Using HPLC and fluorescence detection, ofloxacine, an antimicrobial quinolone derivative was assayed in hair samples from 12 volunteers who had received the drug orally during 1 to 3 days [24]. Once again, hair levels were significantly correlated with drug administration. Amitriptyline and nortriptyline were also detected [25] by GC–MS in electronic impact mode. Chloroquine and its monodesethyl metabolite were detected by negative chemical ionization [26]. In criminal cases, a number of molecules have been detected, including: digoxine by radioimmunological assay (RIA) [27], pentazocine by GC–MS after derivatization using PFPA-PFPOH [28], clozapine by GC–MS in a case of infanticide [29] and clobazam [30]. We recently proposed a very sensitive method for the quantification of detropropoxyphene, a mild analgesic sometimes used by drug addicts because of it costs less than heroin [31].

Therefore, there is a great necessity for a general procedure during police crime investigations for drug screening in human hair. The first approach was made by Couper et al. [32] who described a method for quantifying antidepressants and antipsychotics in post-mortem human scalp hair. However, for all the previously enumerated drugs, various methods have been used in their recovery from hair, including, in the case of drugs of abuse, alkaline digestion, methanol, acid and several enzyme treatments. The alkaline digestion procedure is not suitable for the analysis of benzodiazepines because they are chemically unstable under strong alkaline conditions. This observation made by Couper, can also be applied to numerous other pharmaceuticals and alkaloids of great interest. Flunitrazepam represents a complex problem recently solved by Cirimele et al. [33]. Therefore the procedure suggested by Couper et al. is not satisfactory because it is too restrictive. Considering the need to perform a screening procedure as extensive as possible to all types of toxics, we have developed an efficient, simple and powerful method for the determination of pharmaceuticals in human hair. After sample collection, decontamination and pulverization, powdered hair is incubated in mild acidic or neutral medium followed by a twin solid-phase extraction (SPE) on C_{18} cartridges. Eluates are both analyzed by GC–MS and HPLC–photo diode array detection (PDA). Several examples of application are given as an illustration of the present method.

2. Experimental

2.1. Instrumentation

The gas chromatograph was a 5890 series II plus from Hewlett-Packard (Les Ulis, France), an HP 7673 automatic injector and equipped with the HP 5972 mass selective detector. 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 of 1.3 ml/min. in the constant flow mode (i.e., 60 kPa at 50°C). The temperature of the detector was 300°C.

Splitless injection (1–2 μl) was done at 280°C. The initial oven temperature was 50°C for 2 min and was increased to 310°C at 15°C/min and held for 4.67 min. The chromatography time was 24 min. Repetitive scans were acquired from 40 to 440 u.

HPLC equipment consists of a quaternary gradient pump Waters 600 (Saint Quentin en Yvelines, France), an autoinjector Waters 717 Plus and a PDA UV detector Waters 996. System control, data acquisition and process are made by The Waters Millennium software. The separation column is a Waters Symmetry C_8, 250 mm×4.6 mm I.D. (5 μm particle size) equipped with a 20 mm length guard column (Waters Symmetry C_{18}). Separation conditions were as follows: column temperature 30°C, solvent A= phosphate buffer (pH 3.8), solvent B=acetonitrile; step gradient is 15% B for 6.5 min, then 35% until 25 min, then 80% B for 3 min. Total chromatography duration is 28 min. The equilibration time between two consecutive samples was set at 7 min. The flow-rate of the mobile phase was 1 ml/min for 6.5 min, then increased linearly to 1.5 ml/min from 6.5 min to 25 min and held for 3 min. Re-equilibration was made at 1.5 ml/min. Injection volume varied from 10 to 30 μl. UV spectra from 200 to 350 nm (resolution 1.2 nm) were recorded on-line during the chromatographic run. Solute identification may be automatically performed by comparison of analytical data (retention times and UV spectra), with the 675 pharmaceuticals, pesticides, toxicants and drugs of abuse stored in the personal library.

For SPE, we used C_{18}, 200 mg/3 ml (Isolute) cartridges from IST supplied by Touzart et Matignon (Courtaboeuf, France), and a Vac Elut sample processing station (Analytichem International) from Prolabo (Paris, France).

The ball mill (type MM2) was purchased from Retsch (Haan, Germany).

2.2. Reagents

Methanol, dichloromethane, acetonitrile, hexane, acetic acid glacial, orthophosphoric acid 10%, 0.1 *M* and 0.01 *M* hydrochloric acid, 1 *M* sodium hydroxide, ammonia, sodium bicarbonate ($NaHCO_3$) and anhydrous monobasic sodium phosphate (NaH_2PO_4) of analytical grade were from Carlo Erba (Milan, Italy). Pharmaceutical standards were graciously offered by various manufacturers.

Phosphate buffer 50 m*M*, pH 3.8 of the mobile phase was prepared by dissolving 6 g of NaH_2PO_4 in 1 l of distilled water and adjusting to pH 3.8 with orthophosphoric acid 10%.

The reagent for conditioning the SPE columns was prepared by dissolving 1680 mg of sodium bicarbonate in 100 ml of 10% methanol in water (0.2 *M*), pH 8.6. This reagent was also used as a diluting buffer for the different samples.

Internal standard (I.S.) solution is achieved by dissolving 10 mg of bupivacaine or prazepam free base in 1000 ml of methanol (10 μg/ml).

2.3. Sample collection and decontamination

Collection procedures of hair analysis for drugs have not been standardized. Hair is best collected from the area at the back of the head, called the vertex posterior. This area has less variability in hair growth rate than other areas. Moreover, the hair is less subject to age and sex-related influences [34]. The sample size collected was at least 200–250 mg. When head hair was not available or too short, pubic or axillae hairs were collected.

The problem of external contamination is very important for drugs of abuse as people can be exposed to a smoky or dusty environment. Papers dealing with that problem, which also concerns the establishment of cut-off values, are numerous and are extensively studied by Miezkowski [35,36], Blank [37] and Kintz [38]. The approach is of course very different for pharmaceuticals, as the drugs are supposed to be normally taken by mouth or by another therapeutic route under a normal pharmaceutical presentation. In this case a washing procedure and a degreasing operation is recommended.

Thus, our washing procedure includes two washes with hot water during 3 min followed by two 3 min long washes with dichloromethane.

The samples were cut into segments of 2 cm length which were separately pulverized in a ball mill.

2.4. Drug incorporation

In order to determine the different mechanisms

involved in drug incorporation into hair, Nakahara et al. [39] have extensively studied the intensity of incorporation rate of drugs compared to melanin affinity, lipophilicity and membrane permeability. Their results indicated clearly that both melanin affinity and lipophilicity closely relate to drug incorporation ratios. As an example, there was a thousand times differential of the incorporation ratio between cocaine and its polar metabolite: benzoylecgonine. On the basis of such a study, one understood very well why cocaine, despite a short half-life time, could always be detected in hair at a higher concentration than its metabolites.

Nakahara has also demonstrated that cannabinoids, highly lipophilic drugs, exhibited a poor incorporating ratio. This can be explained as a phenomenon related to the very low permeation of the drug through a bio-membrane due to physicochemical properties of cannabinoids. Therefore, the migration of drugs through membranes depends on their lipophilicity, on the gradient concentration and on the pH gradient on both sides. Since hair is an acidic medium, it was suggested that the pH gradient between blood (pH 7.40) and hair (pH 3.67) [40] might be very important in the flux of an acidic or a basic drug. According to the melanin and acidic protein concentrations contained in white or black hair, it is natural to consider that black hair can concentrate 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 thousand times higher than the one of a basic drug. In fact ketoprofen and niflumic acid were easily identified and quantified by the present method as illustrated at the end of this paper (3.2 and 9.4 ng/mg, respectively). According to the previous comments, the measured concentrations were relatively low with regards to the therapeutic blood concentrations of the drugs (1–5 μg/ml for ketoprofen and 2–35 μg/ml for niflumic acid) [41,42]. On the other side, zolpidem was quantified in the same extract at 2.2 ng/mg with a therapeutic blood concentration ranging only from 0.08 to 0.15 μg/ml [41].

Drug detection of spiked hair is thus very different from hair of drug users. However, 27 (out of 80 tested for the extraction procedure) different drugs were detected and quantified by the present method in real forensic cases.

2.5. Calibration

A stock solution containing 100 μg/ml of each drug free base was prepared in methanol. Drug-free hair samples were collected to realize a standard at 20, 10, 2, and 1 ng/mg of each drugs. 200, 100, 20 and 10 μl of the stock solution of drugs was added to 1 g exactly of weighed powdered hair. After evaporation at ambient temperature of the methanol, the fortified hair was homogenized using the ball mill at high speed for 10 min. Drug-free hair samples were collected from drug-free volunteers. Biological materials were polled, extracted and analyzed by our method. No peaks corresponding to compounds of interest were obtained.

2.6. Extraction procedure

The principal objectives of sample preparation for chromatographic analysis are dissolution of the analytes in a suitable solvent and removal from the solution of as many interferences as possible. There are many preparation techniques that can be used individually or sequentially according to the complexity of the matrix. The use of liquid–liquid extraction is the most common procedure but can be very complex and time consuming e.g., such as the one advocated by Kintz for the analysis of opiates and cocainics [9]. Despite obtaining a very pure extract, one can obtain a quite comparable purity by using SPE. SPE was introduced in the early 1970s and avoids, or minimizes, the disadvantages of liquid–liquid extraction. In toxicology, the most popular columns are represented by combined phases containing reversed-phase C_8, and sulphonated cation-exchange (like Bond Elut Certify). A procedure has been developed for the screening of acidic, neutral and basic drugs in urine by using a copolymeric SPE [43]. Unfortunately, as previously mentioned by Chen et al. [44] data for benzodiazepines were not given. It is for this reason that Chen et al. have extensively investigated the use of Bond

Elut Certify columns in systematic toxicological screening. Since their initial study on 6 drugs [45], the number of compounds tested has been increased to 25. However, the number of different pharmacological classes has not changed and is still very poor. As an example, these drugs include barbiturates (7 drugs), benzodiazepines (5 drugs), drugs of abuse (cocaine, codeine, morphine, amphetamine, methamphetamine and methaqualone), tricyclic antidepressants (2 drugs), carbamate (meprobamate) and miscellaneous (mepivacaine, levallorphan, procaine and promethazine) [44]. So far, very few reports on the screening of various classes of drugs have been published [46]. Moreover, as we have already demonstrated, these columns present no advantages for sample purification and analytes recovery compared to classic C_{18} cartridges [47].

Consequently, rather than a sophisticated protocol on a single mixed phase column, we prefer to apply a twin SPE on C_{18} phase which will enhance the variety of drugs that could be extracted together. Thus, as demonstrated later, in addition to the already listed classes, pharmacological screening will be enlarged to include antimalarials, antipsychotics, β-blocking and β-agonist agents, anti-inflammatory, anticoagulants, antiarythmics and antihistaminics.

Different approaches can be made for hair analysis concerning drugs solvatation prior to sample clean up. Couper et al. [32] have achieved a deep alkaline hydrolysis by complete destruction of the matrix. As already noted, this procedure is not suitable for unstable compounds such as benzodiazepines, opiates, cocainics and cyclopyrrolones. Methanol solubilisation after sonication is already discussed for the analysis of drugs of abuse [48]. Thus, enzymatic treatments and mild acidic solubilisation seem to be the methods of choice for hair pretreatment. As we now have good experience in the use of acidic incubation [31,47], we have retained this procedure for the present technique.

2.6.1. Basic drugs

75 mg of powdered fortified hair or unknown samples were weighed in a conical vial where 30 μl of the I.S. solution were added together with 1 ml of 0.1 *M* hydrochloric acid. After incubation at 56°C for 12 h, the vials are centrifuged at 1500 *g* for 5 min. The supernatant was transferred to a clean vial and 1 ml 0.1 *M* hydrochloric acid was again added to the residue, shaken, centrifuged and the supernatant removed. The two fractions were combined, neutralized with 1 *M* sodium hydroxide and buffered with 2 ml of sodium bicarbonate 0.2 *M* containing 10% methanol (pH 8.6). The columns were conditioned with 4 ml methanol followed by 2 ml bicarbonate buffer. The prepared sample was then applied and allowed to drain under vacuum. The columns were washed with 1 ml water and then 1 ml 10% methanol in water and dried by passing air through for 10 min. The analytes were eluted with three volumes of 500 μl of methanol containing 0.5% of glacial acetic acid. The eluate was evaporated under a stream of nitrogen at 40°C. The residue was dissolved in 20 μl of methanol, 2 μl were injected into the gas chromatographic system while 10 μl were injected into the HPLC system.

2.6.2. Acidic and neutral drugs

75 mg of powdered fortified hair or unknown samples were weighed in a conical vial where 30 μl of the I.S. solution were added together with 1 ml of water. After incubation at 56°C for 12 h, the vials were centrifuged at 1500 *g* for 5 min. The supernatant was transferred to a clean vial and 1 ml water was again added to the residue, shaken, centrifuged and the supernatant removed. The two fractions were combined, and acidified with 2 ml of 0.02 *M* hydrochloric acid.

The columns were conditioned with 4 ml methanol followed by 2 ml 0.01 *M* hydrochloric acid. Prepared sample was then applied and allowed to drain under vacuum. The columns were washed with 1 ml water and then 1 ml hexane and dried by passing air through for 10 min. The analytes were eluted with three volumes of 500 μl of 1% ammoniacal methanol, daily prepared. The eluate was evaporated under a stream of nitrogen at 40°C. The residue was dissolved in 20 μl of methanol, 2 μl were injected into the GC system while 10 μl were injected into the HPLC system.

The incubation step in water gives better results for non-steroidial anti-inflamatory drugs than the incubation in acidic medium. For barbiturates, results are identical in both cases.

3. Results and discussion

The drugs investigated in this study were selected so as to represent various characteristics and classes as well as to cover a wide range of GC and HPLC chromatographic behaviours. Extraction of the drugs can be divided into two groups depending on the pK values of the molecules. However, neutral drugs such as oxazepam, nordazepam, fenfluramine, lidocaine, meprobamate, diclofenac and etodolac can be extracted by both systems. We can make the same distinction between drugs preferentially chromatographed on a GC system or on a HPLC system. For example, meprobamate which does not exhibit any useful UV absorption will be exclusively analyzed on a GC system, while acenocoumarol a non-volatile anticoagulant will be assayed solely by HPLC.

3.1. Determination of recoveries

Ratios of the peak heights of the respective substances to the peak height of the I.S.s were used for calculation. These ratios were compared with those obtained from injections of a mixture of the pure drugs and I.S. in methanol at known concentrations. The concentrations of the drugs in this solution were chosen in such a way that they equalled the concentrations in the reconstituted extracts when the recoveries would be 100%. Using the reported procedure the overall recovery was investigated, including the incubation step for 12 h at 56°C. I.S. for the basic extraction was prazepam while I.S. of the acidic extraction was bupivacaine.

3.2. Benzodiazepines and imidazopyridines

Resuming our work on benzodiazepines and imidazopyridines [49], the present extraction procedure for basic drugs is an adaptation to hair analysis of the previously described method. It has been, in a second step, successfully applied to the analysis of a very wide variety of basic drugs as demonstrated in the following sections.

Table 1 gives the overall extraction recoveries in spiked human hair at 10 ng/mg of each drug, together with the relative standard deviation (R.S.D.) on 10 repetitive measures performed on 10 successive days at the same concentration. The type of extraction: acidic extraction (ASPE) or basic extraction (BSPE) is also given, as is the system of choice for the chromatographic analysis; i.e., GC–MS or HPLC–PDA.

As already debated by Cirimele et al. [33], flunitrazepam (a commonly used hypnotic as an incapacitating agent) cannot be detected by the present method since the concentrations in hair are too low, and thus necessitates the use of a negative chemical ionization detection. However, as shown later in a case report including alprazolam, other benzodiazepines with low therapeutic concentrations can be detected (Section 4.2). As previously described [49], oxazepam, a polar benzodiazepine is incorrectly but fortunately reproducibly retained on C_{18} cartridges

Table 1
Results table for benzodiazepines and imidazopyridine

Compound	Overall extraction efficiency (%)	Between-day precision study ($n=10$) R.S.D.% at 10 ng/mg	Extraction method	Chromatographic method
Alprazolam[ARC]	88.5	8.9	BSPE	HPLC–PDA
Bromazepam	79.4	6.7	BSPE	GC–MS
Clobazam	87.3	9.6	BSPE	GC–MS
Clotiazepam	88.1	6.5	BSPE	GC–MS
Diazepam[ARC]	90.5	7.1	BSPE	GC–MS
Estazolam	86.0	7.9	BSPE	HPLC–PDA
Nordazepam[ARC]	83.2	10.3	BSPE	GC–MS
Nordazepam[ARC]	90.2	5.6	ASPE	GC–MS
Oxazepam	60.1	16.3	BSPE	GC–MS
Oxazepam	89.6	5.9	ASPE	GC–MS
Zolpidem[ARC]	89.7	8.0	BSPE	GC–MS

[ARC] Compounds which were already analyzed in real cases.

under basic conditions. On the contrary, under acidic conditions the recovery is excellent (89.1%).

3.3. Antidepressants: tricyclics and MAOIs

Eight antidepressants were tested including tricyclics and inhibitors of mono amine oxydase (MAOI) which have never been evaluated before. Recoveries ranged from 56.6% for loxapine to 90.1% for amineptine or moclobemide, see Table 2. Recoveries are relatively low concerning some strong lipophilic drugs such as loxapine or dosulepine because the drugs are strongly bound to the C_{18} cartridges and are difficult to elute. As for halofantrine (see below) a complete elution can be easily obtained using three 500 μl volumes of ethyl acetate containing 2% of glacial acetic acid. Nevertheless, considering that other drugs (mild and central analgesics, antiarythmics, β-blocking agents, etc.) are poorly eluted using this latter solvent, the use of acidic methanol seems to be a good compromise.

3.4. Antipsychotics

These drugs have never been evaluated before on Bond Elut Certify columns. Due to a strong affinity to hydrophobic groups, as specified above, the compounds of interest are quite difficult to elute from the stationary phase of a C_{18} solid-phase column. The addition of acetic acid in the elution phase is a necessary condition for a reproducible elution as are the three different fractions which constitute three different fronts of solvent. The use of ethyl acetate containing 2% of glacial acetic acid is also a better choice for the elution of thioridazine and flupenthixol. As mentioned by Couper [32], a liquid–liquid extraction following an alkaline digestion of the hair sample is more efficient than a pretreatment in acidic medium. The same authors estimated that the acidic solubilisation was effective in recovering about 50% of the drugs as compared to alkaline digestion. We personally believe it is underestimated since our relative extraction recoveries ranged from 47.1% for thioridazine to 65.7% for cyamemazine (Table 3). By keeping the hair structure relatively intact, this pretreatment offers the advantage of eluting a cleaner extract with lower interferences [9]. At the very least, alkaline-sensitive molecules are preserved.

3.5. Central analgesics

Pentazocine has been detected in hair in a case studied by Moeller and Fey [28] in which the drug was abused by a medical doctor. Methadone was also reported by the same authors [11]. We have reported a method for the sensitive detection of dextropropoxyphene in hair using the selected ion monitoring mode [31]. In Section 4.4, an interesting case of abuse of pethidine by a medical doctor will be presented (see also Table 4).

3.6. Anticonvulsants

Phenobarbital was already quantified in hair by Goullé et al. [21] who also noticed a group correlation between phenobarbital in hair and blood levels of the drug ($n=23$). Table 5 shows the results for

Table 2
Results table for antidepressants: tricyclics and MAOIs

Compound	Overall extraction efficiency (%)	Between-day precision study ($n=10$) R.S.D.% at 10 ng/mg	Extraction method	Chromatographic method
Amineptine	90.1	6.4	BSPE	HPLC–PDA
Amitriptyline	72.1	8.6	BSPE	HPLC–PDA
Clomipramine[ARC]	61.1	9.1	BSPE	GC–MS
Dosulepine	57.6	10.2	BSPE	HPLC–PDA
Fluoxetine[ARC]	86.2	7.0	BSPE	GC–MS
Loxapine	56.6	11.6	BSPE	HPLC–PDA
Moclobemide[ARC]	90.1	8.8	BSPE	GC–MS
Toloxatone	70.8	9.8	BSPE	GC–MS

[ARC] Compounds which were already analyzed in real cases.

Table 3
Results table for antipsychotics

Compound	Overall extraction efficiency (%)	Between-day precision study ($n=10$) R.S.D.% at 10 ng/mg	Extraction method	Chromatographic method
Amisulpride	67.1	10.7	BSPE	HPLC–PDA
Cyamemazine[ARC]	65.7	7.6	BSPE	HPLC–PDA
Flupenthixol	58.5	7.5	BSPE	HPLC–PDA
Haloperidol[ARC]	70.6	5.2	BSPE	HPLC–PDA
Pipotiazine	65.4	9.8	BSPE	HPLC–PDA
Thioridazine	47.1	11.0	BSPE	HPLC–PDA

[ARC] Compounds which were already analyzed in real cases.

Table 4
Results table for central analgesics

Compound	Overall extraction efficiency (%)	Between-day precision study ($n=10$) R.S.D.% at 10 ng/mg	Extraction method	Chromatographic method
Dextromoramide	73.7	6.3	BSPE	GC–MS
Pentazocine	85.1	10.5	BSPE	GC–MS
Pethidine[ARC]	64.3	5.2	BSPE	GC–MS
Propoxyphene[ARC]	87.1	4.2	BSPE	GC–MS[a]

[ARC] Compounds which were already analyzed in real cases.
[a] Injection was carried out at 190°C and detection was performed in the selected-ion monitoring mode [31].

phenobarbital using the reported technique together with carbamazepine (analysed by HPLC–PDA because of the thermal degradation of the compound), phenytoine and valproic acid. Nevertheless, due to a high molecular polarity, this latter compound has a low and irreproducible recovery which does not permit a correct assay by gas chromatography. Ethosuximide was unsuccessfully extracted.

3.7. Antimalarials

Detection of antimalarials in hair can be a powerful tool for the investigation of drug monitoring in malaria chemoprophylaxis. Chloroquine can be detected by the present method, as can proguanil (both drugs are given in association to French troops stationary in Central Africa) (see Table 6). In areas where the parasite is known to be chloroquine-resistant, mefloquine or halofantrine can be administered. Halofantrine can be extracted from hair at a recovery of 65.1%. For a special study concerning antimalarials, recoveries could be improved, as we have already shown, by the use of 2% acetic acid in ethyl acetate [50].

Table 5
Results table for anticonvulsivants

Compound	Overall extraction efficiency (%)	Between-day precisionstudy ($n=10$) R.S.D.% at 10 ng/mg	Extraction method	Chromatographic method
Carbamazepine[ARC]	90.5	6.5	BSPE	HPLC–PDA
Phenobarbital[ARC]	80.6	5.0	ASPE	GC–MS
Phenytoin	75.6	6.2	ASPE	GC–MS
Valproic acid	30.0	25.6	ASPE	GC–MS

[ARC] Compounds which were already analyzed in real cases.

Table 6
Results table for antimalarials

Compound	Overall extraction efficiency (%)	Between-day precision study ($n=10$) R.S.D.% at 10 ng/mg	Extraction method	Chromatographic method
Chloroquine	91.2	6.3	BSPE	GC–MS
Halofantrine	65.1	10.9	BSPE	GC–MS
Proguanil	89.1	9.6	BSPE	HPLC–PDA
Quinine	77.6	8.2	BSPE	HPLC–PDA

3.8. β-blocking and β-agonist agents

Table 7 lists the different drugs tested by the reported method. β-agonists: terbutaline, salbutamol and clenbuterol (also used in an illicit way as a non-steroid anabolisant by cow-breeders) cannot be detected directly. Due to their difficult chromatographic behaviours, these compounds need a suitable derivatization using BSTFA before GC–MS analysis.

3.9. Drugs of cardiac diseases

In addition to the β-blocking drugs already listed above, antiarythmics, calcium inhibitors, etc., are given in Table 8.

3.10. Alkaloids

Some alkaloids including drugs of abuse were extracted on C_{18} cartridges and results for opiates

Table 7
Results table for β-blocking and β-agonist agents

Compound	Overall extraction efficiency (%)	Between-day precision study ($n=10$) R.S.D.% at 10 ng/mg	Extraction method	Chromatographic method
Atenolol	74.1	7.8	BSPE	HPLC–PDA
Betaxolol	89.6	7.1	BSPE	HPLC–PDA
Clenbuterol[a]	89.2	5.6	BSPE	GC–MS[b]
Salbutamol	70.7	8.2	BSPE	GC–MS[b]
Terbutaline	84.1	7.4	BSPE	GC–MS[b]

[a] This compound was identified and quantified in cow hairs.
[b] After derivatization with BSTFA.

Table 8
Results table for the drugs of cardiac diseases

Compound	Overall extraction efficiency (%)	Between-day precision study ($n=10$) R.S.D.% at 10 ng/mg	Extraction method	Chromatographic method
Amiodarone[ARC]	61.9	8.2	BSPE	HPLC–PDA
Amlodipine	74.2	9.0	BSPE	HPLC–PDA
Clonidine	71.6	8.7	BSPE	HPLC–PDA
Disopyramide	88.2	6.3	BSPE	HPLC–PDA
Flecainide	42.2	15.6	BSPE	HPLC–PDA
Lidocaine[ARC]	89.7	5.1	ASPE	GC–MS
Lidocaine[ARC]	90.2	4.2	BSPE	GC–MS
Verapamil	45.8	13.2	BSPE	HPLC–PDA

[ARC] Compounds which were already analyzed in real cases.

Table 9
Results table for alkaloids

Compound	Overall extraction efficiency (%)	Between-day precision study ($n=10$) R.S.D.% at 10 ng/mg	Extraction method	Chromatographic method
Caffeine[ARC]	90.0	3.5	BSPE	GC–MS
Cocaine[ARC]	86.4	8.9	BSPE	GC–MS
Codeine[ARC]	92.4	4.2	BSPE	GC–MS
Eserine	61.7	6.6	BSPE	HPLC–PDA
Morphine[ARC]	90.7	6.2	BSPE	GC–MS[a]
Sparteine	29.1	17.2	BSPE	GC–MS

[ARC] Compounds which were already analyzed in real cases.
[a] After derivatization with BSTFA.

and cocainics are presented elsewhere [47]. However, due to difficult GC behaviour, morphine and benzoylecgonine have to be derivatized prior to injection (Table 9).

3.11. Anticoagulants

These are very important drugs and can be successfully identified in hair using acidic extraction. In actual fact, acenocoumarol and warfarine can be extracted at 86.1 and 81.1%, respectively, with good precision. These drugs can also be slightly extracted under basic conditions, but at only 15.7 and 12.8% with very poor precision (R.S.D.≥20%) (Table 10).

3.12. Mild analgesics and antiinflammatory drugs

Both derivatives of phenylacetic or phenylpropionic acids are efficiently extracted using the ASPE. Under basic conditions, the compounds can be also slightly extracted allowing in some cases a positive identification even if quantitation is not satisfactory. Table 11 gives different recoveries for paracetamol, floctafenine and antiinflammatory. As a

Table 10
Results table for anticoagulants

Compound	Overall extraction efficiency (%)	Between-day precision study ($n=10$) R.S.D.% at 10 ng/mg	Extraction method	Chromatographic method
Acenocoumarol	86.1	6.2	ASPE	HPLC–PDA
Warfarine	81.2	5.3	ASPE	HPLC–PDA

Table 11
Results table for mild analgesics and antiinflammatory drugs

Compound	Overall extraction efficiency (%)	Between-day precision study ($n=10$) R.S.D.% at 10 ng/mg	Extraction method	Chromatographic method
Diclofenac	91.2	7.4	ASPE	HPLC–PDA
Etodolac	83.7	6.1	ASPE	HPLC–PDA
Floctafenine	78.8	8.7	BSPE	GC–MS
Ibuprofen	72.3	6.0	ASPE	GC–MS
Ketoprofen[ARC]	91.6	4.2	ASPE	GC–MS
Niflumic acid[ARC]	86.3	3.2	ASPE	HPLC–PDA
Paracetamol[ARC]	62.4	7.2	BSPE	GC–MS

[ARC] Compounds which were already analyzed in real cases.

Table 12
Results table for barbiturates and carbamate

Compound	Overall extraction efficiency (%)	Between-day precision study ($n=10$) R.S.D.% at 10 ng/mg	Extraction method	Chromatographic method
Amobarbital	75.7	5.0	ASPE	GC–MS
Meprobamate	87.8	4.2	ASPE	GC–MS[a]
Meprobamate	88.8	5.6	BSPE	GC–MS[a]
Pentobarbital[ARC]	89.2	4.0	ASPE	GC–MS
Thiopental[ARC]	68.5	10.0	ASPE	GC–MS

[ARC] Compounds which were already analyzed in real cases.
[a] Injection was carried out at 190°C.

comparison, using the BSPE, recovery of ketoprofen is only 37.0%, that of ibuprofen 35.8%, diclofenac 40.0% and etodolac 36.5% with standard deviations ranging from 13.6 to 16.5%.

3.13. Barbiturates and carbamate

As for phenobarbital, the procedure for the other representatives of the series is very efficient [51]. Meprobamate can be eluted as well using ASPE as using BSPE (Table 12).

3.14. Miscellaneous

One can find in this section a wide variety of non-homogeneous drugs such as antihistaminics, pesticides, fenfluramine, fluconazole, embutramide and cyclophosphamide (Table 13).

4. Applications

4.1. Case report 1

This case concerns two brothers, two- and three-years old respectively. The boys were discovered dead by their parents in the morning, apparently suffocated by vapours from soft toys which had burned during the night. Blood, urine and hair were taken at the autopsy for toxicological analysis. Although cyanide and carbon monoxide were quantified in appropriate levels to explain the death,

Table 13
Results table for miscellaneous compounds

Compound	Overall extraction efficiency (%)	Between-day precision study ($n=10$) R.S.D.% at 10 ng/mg	Extraction method	Chromatographic method
Acetazolamide	37.6	21.2	ASPE	HPLC–PDA
Albendazole	82.5	5.1	BSPE	HPLC–PDA
Atrazine	47.3	11.2	BSPE	HPLC–PDA
Buclizine	79.0	4.1	BSPE	GC–MS
Cyclophosphamide	90.2	3.1	BSPE	HPLC–PDA
Embutramide	71.5	10.5	BSPE	HPLC–PDA
Endrin	90.6	3.0	BSPE	GC–MS
Fenfluramine	32.4	16.8	BSPE	GC–MS
Fluconazole	78.8	4.2	BSPE	HPLC–PDA
Furosemide	50.0	13.2	ASPE	HPLC–PDA
Ketamine[ARC]	85.6	3.2	BSPE	GC–MS
Metoclopramide	70.7	4.8	BSPE	HPLC–PDA
Nicotine[ARC]	89.2	3.8	BSPE	GC–MS
Levamisole	88.8	4.2	BSPE	HPLC–PDA

[ARC] Compounds which were already analyzed in real cases.

phenobarbital was also discovered in small amounts in urine and blood in both cases (5.4 and 3.9 μg/ml in urine, 2.0 and 1.2 μg/ml in blood, respectively). Since police investigations revealed that the bedroom door was locked from outside by the parents, the judge wanted to know if the barbiturate was intentionally given to the boys the day before or not. In other words, was it usual for them to receive such a tranquilizer or not?

Hair was thus used to solve the problem. A 1.5 cm segment of approximately 100 mg was taken from the vertex posterior at the autopsy. 50 mg of powdered hair was treated for one night at 56°C and extracted according to the ASPE procedure. Phenobarbital was quantified at 1.2 and 1.5 ng/mg, respectively for the two boys (see Fig. 1). Investigations subsequently revealed, that because the children were boisterous, the parents regularly administered such a drug to them.

4.2. Case report 2

This report concerns a 32-year old woman found dead in her bath with neither signs of violence or needle marks found on the corpse. The autopsy report disclosed external and internal signs of visceral congestion. Neither empty boxes of pharmaceuticals or a letter indicating her intention of suicide were discovered. Biological materials were taken for toxicological analysis which concluded an acute poisoning due to zolpidem (hypnotic of the imidazopyridine series) and cyamemazine (antipsychotic of the phenothiazine group). Post-mortem blood concentrations were cyamemazine 4.54 μg/ml and

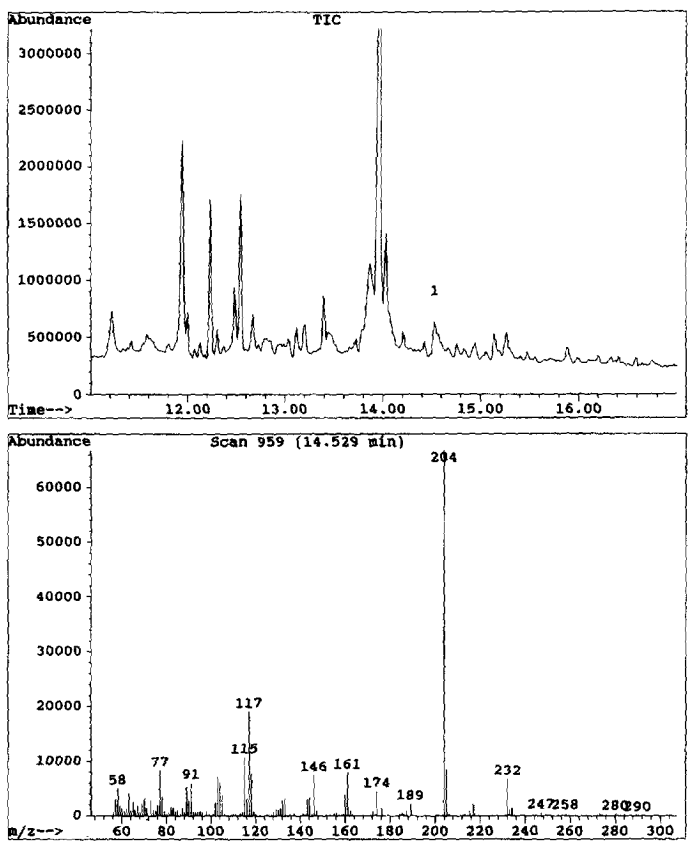

Fig. 1. GC–MS chromatogram of an extract of 50 mg of powdered hair using ASPE procedure. Peak: 1=phenobarbital (1.5 ng/mg) and below mass spectrum of the identified barbiturate.

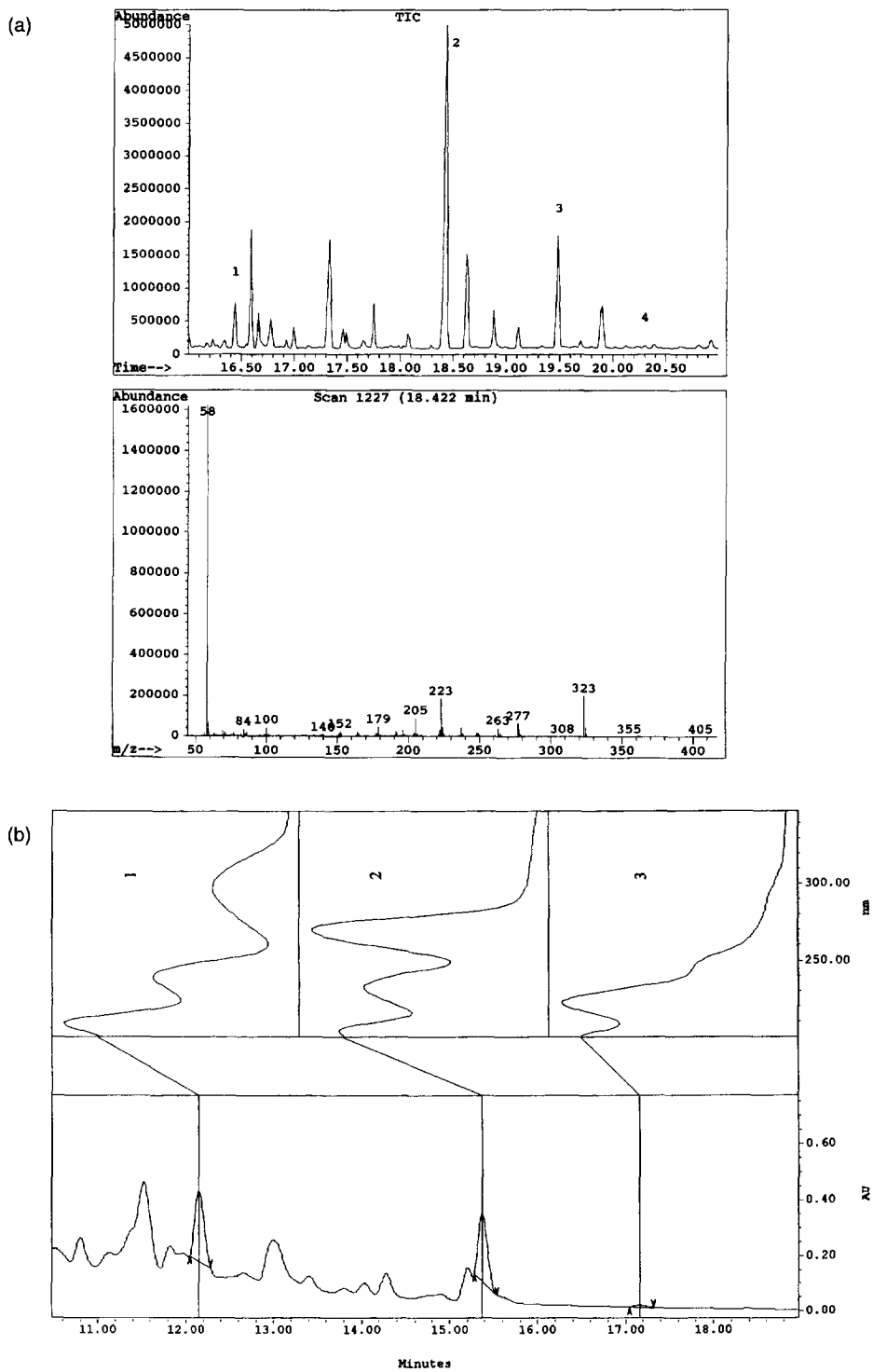

Fig. 2. Chromatograms of an extract of 75 mg of powdered hair using BSPE procedure (a) GC–MS analysis, peaks: 1=moclobemide, 2=cyamemazine (and below its mass spectrum), 3=zolpidem and 4=alprazolam; (b) HPLC–PDA analysis, peaks: 1=zolpidem, 2=cyamemazine and 3=alprazolam.

zolpidem 1.05 μg/ml. Because the treatment prescribed by her physician included alprazolam, zolpidem and moclobemide without any trace of cyamemazine, the reality of a suicide was examined. Was it usual for her to take the drug? Did she regularly take such a treatment and in that case, could she be in possession of such a drug? Hair analysis on a 3 cm long segment of hair taken at the autopsy taken from the sclap enabled us to know all her drug consumption habits. The drug concentrations in her hair were: alprazolam=0.3 ng/mg, moclobemide=4.0 ng/mg, zolpidem=9.7 ng/mg and cyamemazine=11.2 ng/mg (Fig. 2a and b). Her full treatment was therefore known and the theory of suicide was supported.

4.3. Case report 3

The deceased was discovered in an isolated wood, apparently a long time after death. No papers were found in the clothes of the corpse. Main parts of the body had been eaten by small carnivores. A small portion of the skull remained intact but the inferior jaw was absent. The first step in this case was therefore to identify the body. Hair analysis was of great help. A psychiatric hospital situated near the wood, indicated that of one of their patients had disappeared fifteen months beforehand. He had been treated for psychiatric disorders with fluoxetine, alimemazine, nordazepam and additionally received codeine, dextropropoxyphene and paracetamol. On

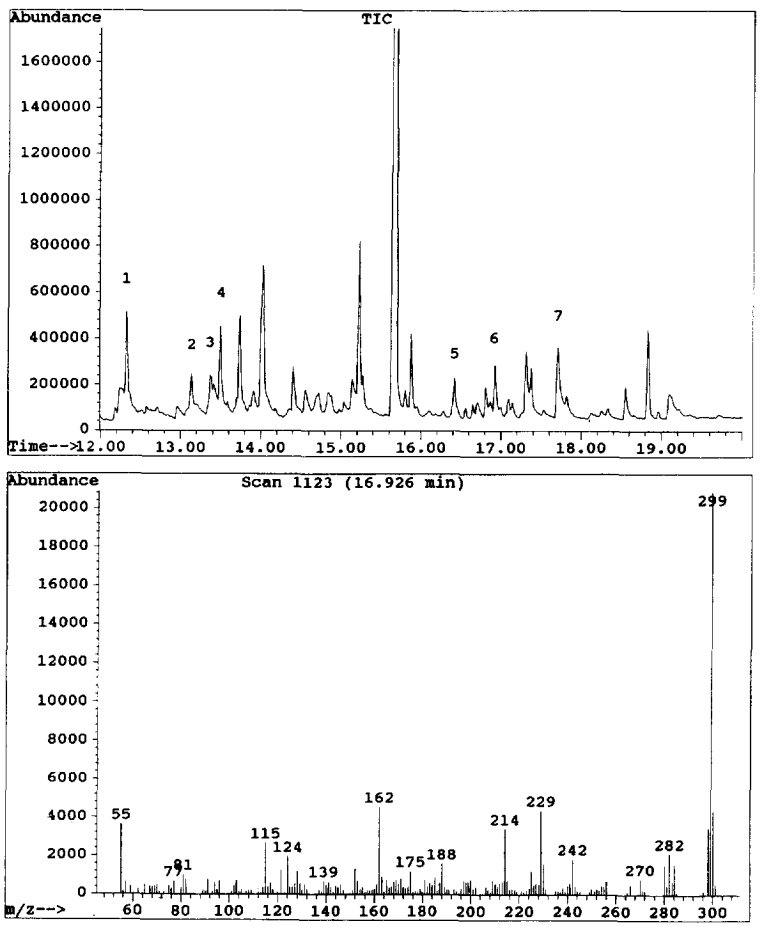

Fig. 3. GC–MS chromatogram of an extract of 75 mg of powdered hair using BSPE procedure. Peaks: 1=dextropropoxyphene artefact (quantitation was realized by an appropriate GC method) [31], 2=caffeine, 3=paracetamol, 4=fluoxetine, 5=alimemazine, 6=codeine and 7=nordazepam.

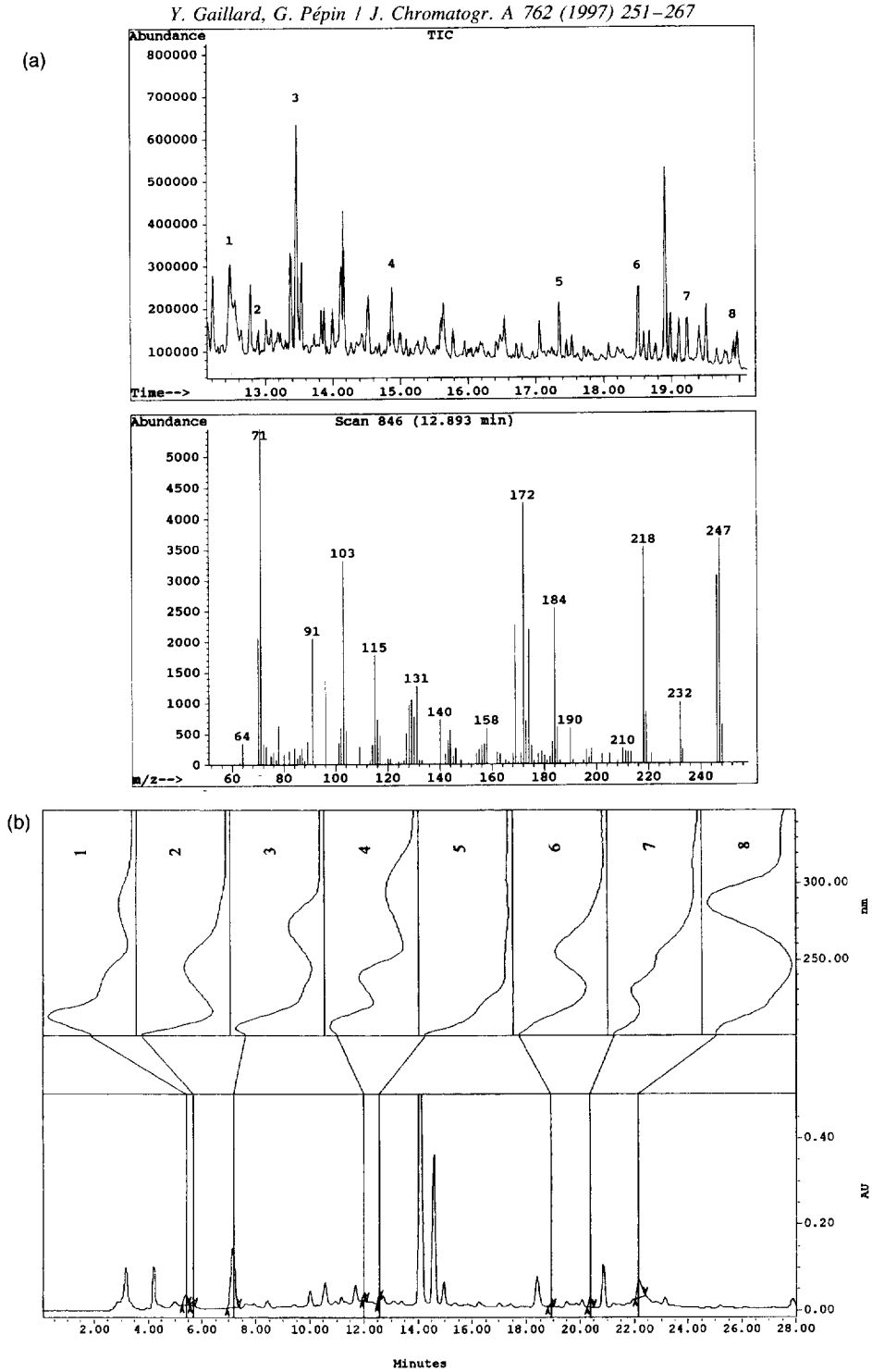

Fig. 4. Chromatograms of an extract of 75 mg of powdered hair using BSPE procedure (a) GC–MS analysis, peaks: 1=paracetamol, 2=pethidine (and below its mass spectrum), 3=caffeine, 4=nefopam, 5=diazepam, 6=promethazine, 7=tiapride and 8=niflumic acid; (b) HPLC–PDA analysis, peaks: 1=tiapride, 2=paracetamol, 3=caffeine, 4=zolpidem, 5=nefopam, 6=ketoprofen, 7=diazepam and 8=niflumic acid.

the undamaged part of the skull a 2 cm segment was taken of his hair. Drugs identified were dextropropoxyphene=2.1 ng/mg (quantified by an appropriate CG method) [31], paracetamol=5.1 ng/mg, fluoxetine=4.3 ng/mg, alimemazine=2.0 ng/mg, codeine=1.7 ng/mg, nordazepam=3.3 ng/mg (see Fig. 3). Oxazepam was not identified. Since the drug has a short half-life time compared to nordazepam, and since it is a polar compound, drug incorporation into hair could be difficult. Detection is certainly not available below a sufficient amount of nordazepam.

4.4. Case report 4

The deceased was a 29-year old female medical doctor. She was known by her husband to abuse diazepam regularly by practicing self infusion of the drug alone or associated to other drugs like nefopam, tiapride or ketoprofene. Her husband discovered the corpse, a needle and syringe which was still standing in her leg. Post-mortem blood concentrations were diazepam=5.0 μg/ml, tiapride=1.9 μg/ml and nefopam=0.06 μg/ml. As there were low blood concentrations to explain this fatality, the syringe was also analyzed. Together with diazepam and tiapride, the presence of morphine was detected. However, this was not detected in the cardiac blood taken at the autopsy. Death could be attributed to a shock of morphine. To assume the validity of such a possibility the blood of the region of the leg should have been analyzed. Unfortunately, it was not taken when the autopsy was performed. In the absence of such a sample we had to know if she was a morphine abuser or if she had used morphine for the first time. Hair analysis on opiates was negative for codeine, morphine and 6-acetylmorphine.

In a second step, to eliminate the possibility that she could have been murdered by her husband, we had to establish the personal drug history of the victim. Did she really abuse diazepam and other drugs? Could she really take morphine corresponding to a harder abusive behaviour? Hair analysis revealed the presence of 10 different drugs corresponding to the usual utilization of 9 pharmaceuticals and one of them was a drug of high abusive potency: pethidine=1.9 ng/mg (see Fig. 4a). The presence of pethidine represents certainly an argument to explain an immediate desire for the consumption of morphine. Other identified drugs were: diazepam (7.2 ng/mg) and nordazepam (0.6 ng/mg), nefopam (6.4 ng/mg), tiapride (8.9 ng/mg), promethazine (5.7 ng/mg), ketoprofen (3.2 ng/mg), niflumic acid (9.4 ng/mg), zolpidem (2.2 ng/mg), paracetamol (3.0 ng/mg) and nicotine, cotinine and caffeine (Fig. 4b).

5. Conclusion

The results of this study indicate that the developed extraction procedure is an effective method in systematic toxicological analysis. The use of an alternative matrix, in this particular case represented by human hair, also appears as a very powerful tool in forensic medicine. The examples presented above illustrate several aspects of the help provided by such an analysis, e.g., the establishment of a personal drug history, psychiatric profile or assistance in body identification.

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