Hair analysis for long-term monitoring of buprenorphine intake in opiates withdrawal

Abstract

Background: Buprenorphine (BUP) is a psychoactive pharmaceutical drug largely used to treat opiate addiction. Short-term therapeutic monitoring is supported by toxicological analysis of blood and urine samples, whereas long-term monitoring by means of hair analysis is rarely used. Aim of this work was to develop and validate a highly sensitive ultrahigh-performance liquid chromatography tandem mass spectrometry method to detect BUP and norbuprenorphine (NBUP) in head hair.

Methods: Interindividual correlation between oral dosage of BUP and head hair concentration was investigated. Furthermore, an intra-individual study by means of segmental analysis was performed on subjects with variable maintenance dosage. Hair samples from a population of 79 patients in treatment for opiate addiction were analyzed.

Results: The validated ultrahigh-performance liquid chromatography tandem mass spectrometry protocol allowed to obtain limits of detection and quantification at 0.6 and 2.2 pg/mg for BUP and 5.0 and 17 pg/mg for NBUP, respectively. Validation criteria were satisfied, assuring selective analyte identification, high detection capability, and precise and accurate quantification. Significant positive correlation was found between constant oral BUP dosage (1–32 mg/d) and the summed up head hair concentrations of BUP and NBUP. Nevertheless, substantial interindividual variability limits the chance to predict the oral dosage taken by each subject from the measured concentrations in head hair. In contrast, strong correlation was observed in the results of intra-individual segmental analysis, which proved reliable to detect oral dosage variations during therapy.

Conclusions: Remarkably, all hair samples yielded BUP concentrations higher than 10 pg/mg, even when the lowest dosage was administered. Thus, these results support the selection of 10 pg/mg as a cutoff value.

Key Words

buprenorphine; hair analysis; UHPLC-MS/MS

Introduction

Buprenorphine (BUP) is a semisynthetic, highly lipophilic opiate derivative. At low doses, BUP acts as a partial agonist at the μ opiate receptor; at higher doses, it presents antagonist effects at the k receptor. BUP is classified as a powerful analgesic (25–40 times more potent than morphine) with prolonged action time. [1–3]

Because of its high potency, the compound is administered at low dosages resulting in low therapeutic serum concentrations (0.5–5 ng/mL).[3]

Since over 10 years, BUP has been used to treat opiate addiction, gradually replacing methadone. [4,5] Being a partial opioid agonist, BUP exerts lower abuse potential and less rapid and intense withdrawal syndrome than full opioid agonists and causes limited respiratory depressant activity. [1,5]

The usual sublingual administration of BUP avoids considerable firstpass metabolism in the liver, otherwise occurring in oral administration. BUP is metabolized by the liver to produce norbuprenorphine (NBUP) and various BUP conjugates, mainly eliminated in the feces or excreted in the urine (30%).[1,6] All the enzymes involved in the metabolic processes exhibit large interindividual variability because of environmental factors and genetic polymorphisms. [7]

In Italy, the Consolidated Law, adopted by the Presidential Decree no. 309 on October 9, 1990 (DPR 309/90), and subsequently amended, provides the legal framework for licit trade, treatment and prevention, and prohibition and punishment of illicit activities in the field of drugs and psychoactive substances. BUP is classified both as an illegal substance (listed in Table I of the DPR 309/90) and a medical drug (listed in Table IIA of the DPR 390/90). The formulations (sublingual tablets) commercially available for opiate addiction treatment are Subutex, a product containing BUP alone, and Suboxone, a BUP/naloxone combination product. Subutex is available at dosages of 0.4, 2.0, and 8.0 mg. Suboxone is produced in 2 dosage forms: 2.0 mg BUP/0.5 mg naloxone and 8.0 mg BUP/2.0 mg naloxone. Naloxone is added to BUP to decrease the appeal of diversion (ie, the use of prescription drugs for recreational purposes) and abuse. [8]

The BUP maintenance therapy to treat opiate addiction is characterized by different phases: (1) the induction phase, in which BUP is typically administered 12–24 hours after abstinence from opiates; (2) the stabilization phase, in which the BUP dose is adjusted for each patient and frequently varied; (3) the maintenance phase, in which a steady dose of BUP is prescribed to the patient. Once stabilization has been achieved, the maintenance period is medically supervised; its duration is individualized for each patient and may be indefinite; (4) reduction stage, in which the BUP dosage is progressively reduced until total suspension.5 Remarkably, BUP doses may vary from 0.8 to 4.0 mg/d in the induction phase and may increase up to 32 mg/d in the maintenance period.[6]

Because of the clinical importance of BUP for the treatment of opiate addiction and the intrinsic complexity of such a treatment, in terms of dosage regulation, toxicological analysis plays an important role to objectively monitor BUP administration. More often, short-term monitoring is supported by toxicological analysis of urine samples. In contrast, hair analysis is less commonly used in the clinical routine control to support long-term monitoring.

Various analytical methods based on gas chromatography–mass spectrometry (MS) or liquid chromatography tandem mass spectrometry (LC-MS/MS) have been developed to detect and

quantify BUP and NBUP in several biological matrices (ie, plasma, serum, whole blood, urine, feces, autoptic specimens, saliva, sweat, and hair samples). [1,7,9–11] As for other toxicological analyses, gas chromatography–MS methods have been progressively substituted by LC-MS–based protocols. Furthermore, highly specific LC-MS/MS methods are progressively substituted by multi-analyte protocols, for both screening and confirmation analysis.[10,12–18]

It is well known that hair analysis allows to monitor drug exposure over a period of several months, unlike blood and urine testing. [11] Thus, the possible occurrence of strong correlation between cumulative hair concentration and daily dosage of BUP, administered in maintenance programs under controlled conditions, may hypothetically represent a precious tool to single out cases of diversion or abuse from the gap between observed versus expected hair concentration.

Only few studies investigated the relationship between the administered dose of BUP and the concentrations of BUP and NBUP in head hair samples. Goodwin et al 2 reported a significant relationship between BUP dose administered to pregnant women and summed hair concentrations of BUP and NBUP, although the scarce number of patients involved in the study limits the significance of these results. More recently, Skoop et al 11 evaluated the reliability of hair analysis to enable an association between BUP and NBUP head hair concentrations and BUP dosage, daily administered to 18 subjects participating in a maintenance program. A reasonable positive relationship was found between observed BUP and NBUP hair concentrations and the daily dose referred to the individual's body weight.

Objective of the present study was to further verify this hypothesis, possibly involving a larger number of patients. Therefore, we developed and validated a highly sensitive ultrahigh-performance liquid chromatography (UHPLC) MS/MS method to detect BUP and NBUP in head hair to investigate the interindividual correlation between oral dosage of BUP and hair concentration. Furthermore, an intra-individual study by means of segmental analysis was performed on subjects with variable maintenance dosages to verify the applicability of segmental hair analysis in revealing oral dosage variations during therapy.

Materials and Methods

Chemicals and Reagents

BUP, NBUP, naloxone, BUP-d4, and NBUP-d3 were purchased from LGC Promochem SRL (Milan, Italy). Methanol, acetonitrile, and ammonium formate were provided by Sigma–Aldrich (Milan, Italy). Formic acid (LC–MS grade) was obtained by Fisher Scientific (Geel, Belgium). Ultrapure water was obtained using a Milli-Q UF-Plus apparatus (Millipore, Bedford, MA). Standard stock solution was stored at -20°C until used. The deuterated compounds BUP-d3 and NBUP-d4

were used as the internal standards (IS). Two working solutions were prepared by dilution in methanol at final concentrations of 1 and 4 μg/mL for BUP and NBUP, respectively. Lastly, the IS working solution was prepared in methanol at a final concentration of 1 μg/mL.

Study Protocol

Seventy-nine subjects (10 women and 69 men, aged 23–61 years, mean = 43) undergoing drug treatment were involved in this study. Head hair samples were collected over a 12-month period from 2 Abuse Treatment Services located in Torino (Piedmont, Italy). Careful selection of subjects was made to involve only patients with a perfectly known Suboxone administration history. General information on their gender, age, hair color, weight, and height is listed in Table 1. Personal interview declarations and medical history were also collected, including information about the use of cosmetic products and both recent and previous intake of other legitimate and illicit drugs.

All subjects had been receiving BUP by the sublingual route for over 6 months, at least. They received the oral doses on a weekly basis at the Abuse Treatment Services, where every time they underwent medical examinations and urine testing for opiates.

To investigate an interindividual correlation between oral dosage of BUP and head hair concentrations, only patients who took a constant dosage (ranging from 1 to 32 mg/dye) for the last 3 months were selected (Table 1). The Pearson correlation coefficient was computed to estimate the significance of the dose–concentration relationship. The software package SPSS (SPSS Inc, Chicago, IL), version 20.0, for Windows has been used for calculations. Fifteen subjects (cases 38–52 in Table 1) who declared an irregular intake of BUP were considered separately.

To investigate an intra-individual correlation between BUP concentrations in segmented hair samples and variations of the daily BUP intake, only patients (cases 53–64 in Table 1) who scaled up or down constant BUP daily dosages (during extended periods of at least 1 month) were selected (eg, patient of case 57 consumed 8 mg/d BUP for 3 months and then scaled down to 4 mg/d BUP and maintained this dosage for another 3 months).

The remaining patients (n = 15) were excluded because indubitable information about effective BUP intake was not available.

Only head hair samples were collected from each subject. All hair samples were cut from the posterior vertex as close as possible to the scalp, using freshly disinfected scissors. The samples were stored in closed containers at room temperature until analysis. For subjects in maintenance program (constant dosage of BUP administered), only the proximal segments, 0–3 cm, were analyzed whenever a longer head hair sample was collected. Shorter head hair samples were analyzed in their full length. Medical records were carefully verified to ensure that the analyzed hair segments matched the periods in which BUP was administered at constant dosage. For the subjects whose daily BUP dosage was changed, a 6-month period was investigated utmost;

therefore, proximal segments up to 6 cm (total length) were analyzed whenever a longer head hair sample was collected. Shorter head hair samples (at least 1 cm) were analyzed in their full length. A growth rate of 1 cm/mo for human scalp hair 19 was considered to separate the segments matching the BUP intake variation.

The study protocol was approved by the recognized Ethics Committee at San Luigi Gonzaga University Hospital (Torino, Italy). All patients provided written informed consent before attending the study, and an anonymous code was attributed to each participating subject to respect privacy regulations.

Hair Sample Treatment

Hair samples were washed twice with methylene chloride and methanol (2 mL, 3 minutes) in sequence and then dried. Each sample was cut into small pieces (1–2 mm length) and weighted. About 50 mg of hair was added with BUP-d4 and NBUP-d3 (200 pg/mg final concentration) and 1 mL of NaOH (1 mol/L). The samples were multimixed for 2 minutes, then centrifuged (10,000g, 3 minutes), and then incubated overnight at room temperature. Fifteen hours later, 1 mL of HCl (1 mol/L) was added. The samples were multimixed for 2 minutes. Two milliliters of carbonate buffer (100 mmol/L, pH 9.6) was added, and then a liquid–liquid extraction with hexane/chloroform/propanol (vol:vol:vol, 6:3:1) was performed. The samples were multimixed for 5 minutes and then centrifuged (10,000g, 5 minutes). The organic supernatant was separated and dried under nitrogen at 70°C. The residue was dissolved in 50 µL of methanol, and the resulting solution was transferred into a clean vial, from which 2 µL was injected into the UHPLC-MS/MS instrument.

UHPLC-MS/MS Protocol

Analyses were performed using an Agilent 1290 Infinity LC system (Agilent, Palo Alto, CA), interfaced to a QTRAP 4500 Mass Spectrometer (AB Sciex, Darmstadt, Germany) equipped with an electrospray Turbo Ion source operating in positive ion mode. A Shim-pack XR-ODS C18 column (75 × 2.0 mm internal diameter, 1.6 µm particle size), protected by C18 guard column, was used for the chromatographic separation.

The column oven was maintained at +45°C, and the elution solvents were 0.1% water/formic acid/2 mmol/L ammonium formate (solvent A) and 0.1% acetonitrile/formic acid/2 mmol/L ammonium formate (solvent B). The mobile phase eluted under the following linear gradient conditions (A:B, vol:vol): from 98:2 to 60:40 in 2.0 minutes to 10:90 in 0.5 minutes, followed by isocratic elution at 90% B for 1.0 minute. The flow rate was 0.5 mL/min and the total run time was 6.5 minutes, including reequilibration at the initial conditions. The mass analyzer operated in the

selected reaction monitoring (SRM) mode. To establish appropriate SRM conditions, optimization of the mass spectrometer was conducted by direct infusion of the analytes into the electrospray ionization capillary and the declustering potential was adjusted to maximize the intensity of the protonated molecular species. For each SRM transition, the collision offset voltage values and the cell exit potentials were also optimized. Each SRM transition was maintained during a time window of ±20 seconds around the expected retention time of the corresponding analyte, and the SRM target scan time (ie, sum of dwell times for each SRM cycle) was 0.18 seconds, including pause times of 5 milliseconds between consecutive SRM transitions. The best results were obtained using a source block temperature of +550°C and an ion-spray voltage of +2000 V. Nitrogen was employed as the collision gas (5 × 103 Pa). The gas settings were as follows: 35.0 psi curtain gas, 8.0 psi collision gas, 35.0 psi ion source gas (1), and 50.0 psi ion source gas (2). The Analyst 1.6.1 (AB Sciex) software was used for data processing. All analytes and IS, their corresponding retention times (tR), SRM transitions, and potentials are presented in the Supplemental Digital Content 1 (see Table S1, http://links.lww.com/TDM/A87).

Validation

The analytical method was validated for BUP and NBUP in accordance with national and international guidelines.20–22 For BUP and NBUP, the following parameters were investigated: selectivity, linearity range, limit of detection (LOD), limit of quantification (LOQ), imprecision, inaccuracy, carryover, and matrix effect phenomena. Selective identification criteria were used for mere qualitative determination of naloxone, as it was known that all patients were treated with Suboxone.

Blank head hair samples were collected from 5 healthy volunteers (laboratory personnel) and used as the working matrix for all validation experiments.

Identification criteria for the analytes were established according to national and international guidelines. [20–22] For each analyte, the signal-to-noise ratio (S/N) was measured for the corresponding mass transitions at the expected retention time windows. An S/N <3 was considered satisfactory to verify method selectivity.

Retention time precision was determined at 5.0 and 500 pg/mg BUP concentrations and at 20 and 2000 pg/mg concentrations for NBUP. Deviation of 1%–2% from calibrators and controls is acceptable for LC-based methods. Two qualifier transitions were monitored, in addition to the primary fragmentation, as reported in the Supplemental Digital Content 1 (see Table S1, http://links.lww.com/TDM/A87). Variations of relative transition intensities were considered acceptable within ±20%, with respect to the control. Their repeatability was determined on 5 blank head hair samples spiked with 5.0 and 500 pg/mg of BUP concentrations (20 and 2000 pg/mg for NBUP). The S/N was measured on all mass transitions at the expected analytes' retention time.

Analogous check was made on blank samples spiked with the IS only, to verify that the isotopically labeled standards did not contain a significant concentration of the nonlabeled analytes as impurities.

The linear calibration model was checked by analyzing (2 replicates) blank head hair samples spiked with working solutions at 7 final concentrations, using BUP-d4 and NBUP-d3 as IS. Linearity was evaluated over the 5.0–500 pg/mg range for BUP and 20–2000 pg/mg for NBUP. The linear calibration parameters were obtained using the least squares regression method. The squared correlation coefficient (R2) was used to roughly estimate linearity. The appropriateness of the linear model was assessed by performing linear lack-of-fit test and residual plots analysis. The assumption of homoscedasticity and the significance of slope and intercept of the regression line were successfully verified.

LOD was estimated as the analyte concentration, the response of which provided S/N = 3, determined by the least abundant ion. Numerical value of LOD was extrapolated from S/N value of the lowest concentration level using the calibration curve. The noise was measured from 0.05 minutes before the peak onset till the beginning of the peak. Similarly, LOQ was estimated as the analyte concentration that yielded an S/N ratio >=10.

Imprecision and trueness (expressed as percent coefficient of variation, CV%, and percent bias, respectively) were evaluated by analyzing 5 head hair samples spiked at 2 concentrations (5.0 and 500 pg/mg for BUP; 20 and 2000 pg/mg for NBUP). Standard criteria for quantitative methods are generally regarded as satisfactory when assay imprecision is below 15%–20% for all concentrations, whereas trueness is considered satisfactory when the experimentally determined concentrations lie within ±15% from the expected values.

The matrix effect was calculated as the mean value obtained from 5 blank head hair samples (from different individuals). Hair samples were spiked after the extraction step at the final BUP concentrations of 5.0 and 500 pg/mg (at 20 and 2000 pg/mg for NBUP). For each analyte, the chromatographic peak areas were compared with the mean peak areas of 3 standard solutions prepared in methanol, that is, the solvent used to dissolve the sample residues to be injected into the UHPLC.23 The variability of matrix effect among different hair samples was expressed as percent bias (average of 5 replicated).

For carryover evaluation, the background chromatographic profiles for each analyte were monitored during the analysis of blank hair samples injected for 5 times after the chromatographic run of a spiked blank hair sample containing BUP at 600 pg/mg and NBUP at 2400 pg/mg concentrations. To assure the absence of carryover, the S/N for each transition had to be lower than 3.

The extraction recovery was not calculated because all validation experiments were performed on spiked blank hair samples.

Results and Discussion

UHPLC-MS/MS Protocol and Validation Results

The optimized UHPLC-MS/MS method allowed a concurrent quantitative determination of BUP, NBUP, and 2 IS, plus the qualitative identification of naloxone, in 6.5 minutes of total chromatographic run. Retention times were 1.2, 2.0, and 2.4 minutes for naloxone, BUP, and NBUP, respectively (see Table S1, Supplemental Digital Content 1, http://links.lww.com/TDM/A87). Figure 1 shows the SRM chromatograms recorded from blank head hair samples spiked with BUP at 10 pg/mg and NBUP at 40 pg/mg. Three SRM transitions are depicted for each analyte and 1 transition for the IS.

The imprecision for retention times, measured at low and high concentrations (first and last point of the linearity range), showed random fluctuations within ±1.0%, confirming their repeatability at both concentrations.

For each analyte, the relative abundance of the 3 selected SRM transitions was found to vary by less than ±8% (CV%). Again, this variability (<20% in absolute value) meets the requirements for the unambiguous identification of all analytes included in the assay.

Linear calibration was observed for BUP and NBUP over the ranges 5.0–500 and 20–2000 pg/mg, respectively. Squared correlation coefficients (R2) were equal to 0.9996 and 0.9998 (Table 2). All back calculations of calibrators were within ±15% at each calibration level.

LOD values were 0.6 and 5.0 pg/mg for BUP and NBUP, respectively. The corresponding LOQ values were 2.0 and 17.0 pg/mg (Table 2). Positive detection (S/N > 3) of all analytes at their approximate LOD concentrations was confirmed experimentally.

Accuracy and precision requirements were satisfied: the percent bias and the CV% were lower than ±6.0% at each calibration level (Table 2).

For BUP and NBUP, the average matrix effect never exceeded ±20%, expressed as percent bias (Table 2).

No carryover effects were observed under the conditions described in the experimental section. For the blank head hair samples, the S/N ratios were always lower than 3 at the retention times expected for BUP and NBUP.

Dose–Concentration Relationship: Interindividual Study

Table 3 shows the experimental results for the patients after the maintenance program (cases 1–37) that entail constant BUP daily dosages and the patients with irregular BUP daily intake (cases 38–52). The length of the analyzed segments, oral BUP intake, and concentrations of BUP and NBUP, their sum, and ratio values are reported for each patient.

Over the entire investigation period, all patients underwent urine testing on a weekly basis to test for opiates. With the exception of case 52, all urine samples resulted negative. A parallel investigation of opiates in hair was not performed in this study because of the insufficient quantity of hair available (after analysis for BUP determination).

Over the range of 1–32 mg/d, the median (interquartile range) value of BUP head hair concentration is equal to 73 (78) pg/mg. For NBUP, the median (interquartile range) value is 502 (516) pg/mg. The average ratio between NBUP and BUP is equal to 7.4 ± 3.8. The extensive polymorphism occurring in CYP3A4 may explain the high degree of interindividual variability in measured hair concentrations of BUP and NBUP that has been observed among subjects who received the same BUP dose.[11] In this study, all NBUP concentrations were higher than BUP concentrations, as was found in previous independent studies,11,24,25 whereas some other studies reported BUP/NBUP ratios to be higher than 1 in hair samples.1,26–28 No dependence of ratio values on gender, age, body mass index, and hair color, as possible factors for bias, was evident from the data. Because the incorporation of the drugs in hair depends on their lipophilicity, greater concentrations of the parent drugs are usually expected in comparison with that of metabolites. Nevertheless, Kuhlman et al reported NBUP/BUP ratios >1 in plasma samples for subjects who constantly consumed BUP, and this outcome was used to support the same experimental evidence in hair samples. [25]

Specific studies have been performed to clarify the reason why discrepancies in the NBUP/BUP ratio were found in the literature. The direct comparison of all these results is complex because the published studies refer to different populations, designs of experiments, and analytical protocols for hair analysis. Nevertheless, hair decontamination and digestion were suggested as possible factors of the NBUP/BUP variation. For example, greater loss of BUP than NBUP was found after double decontamination with dichloromethane and that could explain why the remaining concentration in hair samples (collected from 33 subjects) was higher for NBUP than for BUP.26 Indeed, opposite results (BUP concentration > NBUP concentration) were obtained for 60% of the same samples if the concentrations found in hair and in decontamination solutions were summed up.26 Upon acidic or alkaline digestion, the conversion of BUP into NBUP may bias the ratio values, even though this does not affect the quantitative interpretation of the results when the summed up BUP and NBUP concentrations are considered.

In most cases, BUP concentrations were higher than 10 pg/mg, including the cases when the lowest dosage was administered (1 mg/dye), as reported in Table 3. Only 2 patients (44 and 52) had head hair concentrations of BUP <=10 pg/mg. Supplemental Digital Content 2 (see Figure S1, http://links.lww.com/TDM/A88) shows the SRM chromatogram for case 44 that presented the lowest sum of BUP and NBUP hair concentrations (31 pg/mg). Case 44 relates to a woman who regularly took 6 milligrams per dye of BUP for more than 3 months before hair sampling. The keratin structure of her hair was visibly damaged and dry; the patient confirmed that she frequently

dyed her hair using commercial products. It has been reported in the literature that severe damage of the keratin matrix may justify a significant loss of drug from the hair,19,29 as possibly occurred in this case. In fact, nondamaged head hair collected from other patients who regularly assumed 6 milligrams per dye BUP presented concentrations ranging from 38 to 149 pg/mg (Table 3). Case 52 relates to a male who started the replacement therapy with 32 milligrams per dye of BUP just few days before hair sampling. Clinicians suspected previous occasional consumption of BUP, without prescription. Accordingly, a concentration of 9 pg/mg (Table 3) was found in his 3-cm hair sample, which cannot be justified by his recent oral BUP intake within drug replacement therapy. Further analyses were planned to monitor the expected increment of BUP concentration in head hair during therapy, but the patient died of opiate overdose 1 week after the first hair sampling.

Remarkably, the experimental results presented in Table 3 support the choice of 10 pg/mg as a reliable cutoff value to discriminate regular dosage administration from occasional intake, as previously suggested. [20,30]

The data collected from the first 37 hair samples listed in Table 3 were used to evaluate the possible occurrence of a dose–concentration relationship for BUP detected in hair samples. The time window was limited to the last 3 months (about 0–3 cm proximal segments). Figure 2A shows the BUP head hair concentrations plotted against the oral BUP doses (1–32 mg/dye). Figure 2B shows the relationship between the oral dosages and the summed BUP and NBUP concentrations. A significant positive dose–concentration relationship was found, as suggested by Skopp et al 11 and Goodwin et al.2 The Pearson coefficients were equal to 0.86 ($P < 0.001$) and 0.83 ($P < 0.001$), respectively, when only BUP or summed BUP and NBUP concentrations are considered. Nevertheless, the presence of a severe interindividual variability limits the chance to use this mathematical relationship to predict the oral dosage taken by each subject from either the measured BUP or the summed BUP and NBUP concentrations in head hair. Indeed, the inaccuracy (expressed as percent bias) between predicted and measured summed BUP and NBUP concentrations ranges from -67% (case 7 at 3.5 mg/dye) to +637% (case 26 at 8 mg/dye). The consequent inaccuracy on the X values (oral BUP dosage) ranges from -93% to +242%, resulting in an enormous underestimation or overestimation of the daily BUP intake. Similarly, the inaccuracy calculated from measured BUP concentrations ranges from -87% to +146%.

It has been suggested that possible sources of bias may arise from gender, body mass index, hair color, ethnicity, metabolism, pathological conditions, assumption of other xenobiotics (eg, medicines or illicit drugs), contribution from sweat, and environmental factors. Clearly, further sources of bias and unpredictability may arise from the inaccuracy of self-reported dosage intake. To date, several studies were planned to investigate the effect of hair color on drug concentrations. As BUP is a cation at physiologic pH, the drug is expected to be incorporated into pigmented hair to a greater extent than into nonpigmented hair.[11] Greater deposition of BUP in pigmented hair was actually verified with rat models. This and other similar studies proved that color (ie, melanin

content) plays a role in the accumulation of drugs in hair. On the other hand, Mieczkowski et al 31 pointed out that (1) human hair coloration is not similar in its morphology and physiology to the reticulated pattern of black- and white-furred animals, (2) hair color may greatly vary within a single subject and even along the shaft of a single hair, and (3) melanin represents a minor fraction of the total hair mass (<1%). Thus, the effect of hair color on the complex process of drug accumulation seems to be far from being statistically relevant.

In this study, the number of samples considered is too limited to further investigate all these factors on a statistical basis. Besides, different mathematical models built by dividing the subjects into subcohorts in which some of these factors are kept constant would have little practical application with respect to a simpler rough correlation model that just relates head hair concentrations and BUP oral dosages for a wide cohort of patients undergoing opiate withdrawal treatment, especially considering that many of these factors are circumstantially and virtually never measured or known. [31]

Contrarily, the use of cosmetic hair products is nowadays considered a major source of bias. Thus, in hair analysis of drugs of abuse, the cosmetic history of a hair sample should always be considered.32 Ingredients contained in cosmetic products and the reactions occurring during their application can relevantly affect drug concentrations in hair. [31,32] For example, diacetylmorphine readily hydrolyzes at basic pH values, whereas morphine was observed to be partly converted to morphinone and pseudomorphine in the presence of an oxidizing agent. Many recent evidences show that the structure and the physicochemical properties of the keratin matrix are deeply changed after cosmetic treatments, and this essentially leads to a decrease in drug incorporation and conservation capacity. [32]

Although a strict relationship between dose and head hair concentrations cannot be drawn from the data, a discriminating line between low and high oral BUP dosages could be proposed around 10.0 milligrams per dye. Summed BUP and NBUP hair concentrations lower than 1500 pg/mg were found for all patients who assumed less than 10.0 BUP mg/d, whereas values larger than 1500 pg/mg were found for 7 patients who assumed more than 15 mg/d of BUP out of 10.

Further 15 hair samples collected from patients with irregular administration of BUP (cases 38–52) were analyzed. Rather scattered values for the analytes under study were obtained (Fig. 3). For the 9 patients (cases 38–46) hypothetically after a replacement treatment with an oral daily intake of BUP lower than 10.0 pg/mg (Table 3), most BUP and NBUP concentrations were found to be quite low, but with the notable exception of cases 40 and 43, showing a summed BUP and NBUP concentration above 1500 pg/mg (Fig. 3). Unlikely, subjects supposedly assuming 16–24 mg of BUP/d (cases 47–52) all exhibited BUP and NBUP hair concentration in the low range. As a matter of fact, they all declared to either habitually take a lower daily dosage than prescribed or entirely skip the assumption several times a week, so as to preserve extra BUP doses for periods of anxiety.

Dose–Concentration Relationship: Intra-Individual Study

Patients 53–64 had the prescribed BUP dosage varied during the investigated period. Their hair was segmented, and the segments were analyzed separately. Results from 2 or 3 segments were compared for each patient. Table 4 reports in detail the length of the analyzed hair segments, the oral BUP dosages, BUP, NBUP, and summed concentrations, together with the percent variation between the BUP doses assumed in different time periods and the corresponding changes between the summed BUP and NBUP concentrations. These differences were calculated so that up-variations (positive values) corresponded to an increment of the dosage with time, in turn referring to segments progressively closer to the scalp.

Although the number of samples is limited, a strong correspondence is observed between variations of doses and head hair concentrations (summed BUP and NBUP values) in the time-matching segments. In 10 cases out of 12, a closely proportional difference is evident. Only for case 60, the percent difference is not significant, considering the analytical error associated with the hair concentration values. Although preliminary, these results apparently indicate that hair segmental analysis could be reliably used to show intra-individual dosage variations during therapy while different patients taking the same dosage showed considerably different drug incorporation degrees into the hair. For example, the data reported in Table 4 indicate that the hair collected from 4 subjects who assumed 16 mg of BUP/d present summed BUP and NBUP concentrations ranging from 833 to 2909 pg/mg.

Conclusions

Nowadays, segmental hair analysis is often adopted to draw information on cumulative drug exposure over time, on the period of drug use, or to depict a chronological sequence of drug exposure. Although there is a broad agreement that qualitative results from hair analysis are truthful, the interpretation of quantitative results is still under debate,[19,33[for example, to predict the daily intake of an administered drug from its concentration in hair.

In this study, qualitative detection of BUP, NBUP, and naloxone was clearly evident in the hair of all patients subjected to Suboxone maintenance program. In contrast, quantitative results showed only a modest correlation between dosage and hair concentration, not sufficient to allow a reliable prediction of real dose intake of Suboxone. Indeed, high interindividual variability biased the head hair concentrations, likely depending on the different incorporation degrees of the drug into the keratin matrix. A threshold value of 1500 pg/mg (summed BUP and NBUP concentrations) can nevertheless be suggested to roughly distinguish the subjects as taking either low (<10.0 mg) or high daily dosage (>10.0 mg) of BUP.

On the other hand, quantitative results proved to be reliable when intra-individual segmental hair analysis is performed to depict a chronological scheme of a drug exposure. Indeed, the summed up concentrations of BUP and NBUP in head hair reproduced, on a relative scale, the dosage that each individual patient regularly takes. Because it is frequent that the patients do not take the entire prescribed BUP dosage, especially during a long period of maintenance, the opportunity envisioned by the present study to objectively evaluate intra-individual variations of BUP intake based on toxicological hair analysis is of relevance. Indeed, even though the hair incorporation phenomenon is complex and many factors may increase data variability, drug concentration in hair is the result of a cumulative process and may provide a more reliable information during a maintenance program than that coming from other biological matrices, such as urine.

Lastly, it is worth noting that the quantitative results herein discussed support the selection of 10 pg/mg as a cutoff value to discriminate between regular dosage administration and occasional intake.[20,30]

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Image gallery

Case	Gender	Age (yrs)	Weight (kg)	Height (cm)	Hair Color	Cosmetic Treatments	Hair Length (cm)
Patients in maintenance program with constant BUP doses							
1	M	50	NA	NA	White	No	3.0
2	M	51	65	173	Brown	No	4.0
3	M	40	80	182	Brown	No	26
4	M	33	73	168	Black	No	3.5
5	M	46	53	169	Black	No	6.0
6	M	43	63	166	Brown	No	2.0
7	M	42	NA	NA	Black	No	5.0
8	M	43	74	169	Black	No	3.0
9	M	54	70	170	White	No	3.0
10	M	49	63	168	Gray	No	12
11	M	41	75	170	Brown	No	24
12	F	29	50	157	Blond	Yes (dye)	26
13	F	49	66	166	Red	Yes (dye)	30
14	M	50	NA	NA	Brown	No	3.5
15	M	47	NA	NA	Black	No	3.5
16	M	46	NA	NA	Gray	No	4.0
17	M	NA	70	172	Brown	No	4.0
18	F	45	NA	NA	Blond	Yes (dye)	7.0
19	M	39	NA	NA	Black	No	39
20	M	35	35	170	Brown	No	3.0
21	M	52	78	172	Black	No	6.0
22	M	40	85	186	Gray	No	1.5
23	M	36	68	174	Gray	No	3.5
24	M	61	85	183	White	No	2.5
25	M	38	89	180	Brown	Yes (dye)	4.0
26	F	43	68	165	Blond	Yes (dye)	11
27	M	52	61	173	Brown	No	29
28	M	40	90	190	Black	No	4.5
29	M	47	72	172	Gray	No	20
30	M	47	88	173	Black	No	2.0
31	M	38	84	178	Black	No	4.5
32	M	54	58	171	Gray	No	2.5
33	M	51	90	170	Gray	No	5.0
34	M	35	65	170	Gray	No	2.5
35	M	43	95	175	Brown	No	14
36	M	43	115	168	Black	No	3.0
37	M	42	95	172	Brown	No	4.0
Patients with irregular BUP daily intake							
38	M	38	NA	NA	Black	No	2.0
39	M	44	60	165	White	No	4.0
40	M	29	66	169	Brown	No	7.0
41	M	43	127	176	Black	No	2.5
42	M	41	87	182	Brown	No	5.0
43	M	42	70	175	Black	No	2.5
44	F	44	47	160	Blond	Yes (dye)	18.5
45	M	50	90	175	Brown	No	1.0
46	F	33	54	165	Black	No	6.5
47	M	43	75	180	Gray	No	39
48	M	54	80	172	Gray	No	4.5
49	M	54	120	175	White	No	6.5
50	M	39	NA	NA	Black	No	2.5
51	M	38	106	178	Black	No	4.5
52	M	54	NA	NA	White	No	3.5

TABLE 1 -a For Each Subject, Gender, Age, Weight and Height, Hair Color, Hair Cosmetic Treatments, and the Length of Measured Hair Segments Are Listed

TABLE 1. (*Continued*) For Each Subject, Gender, Age, Weight and Height, Hair Color, Hair Cosmetic Treatments, and the Length of Measured Hair Segments Are Listed

Case	Gender	Age (yrs)	Weight (kg)	Height (cm)	Hair Color	Cosmetic Treatments	Hair Length (cm)
Patients in variable maintenance program (hair segmental analysis)							
53	F	40	80	174	Black	No	31
54	M	38	61	172	Brown	No	4.0
55	M	44	70	165	Black	No	10.5
56	M	39	68	170	Brown	No	4.0
57	F	40	52	164	Brown	No	21
58	M	46	80	170	Brown	No	29
59	M	49	67	170	Black	No	7.0
60	M	45	65	170	Brown	No	4.5
61	M	44	NA	NA	Black	No	3.0
62	M	52	NA	NA	Black	No	6.0
63	M	53	NA	NA	Gray	No	7.0
64	M	52	NA	NA	Black	No	4.0

NA, not available.

TABLE 1 -b For Each Subject, Gender, Age, Weight and Height, Hair Color, Hair Cosmetic Treatments, and the Length of Measured Hair Segments Are Listed

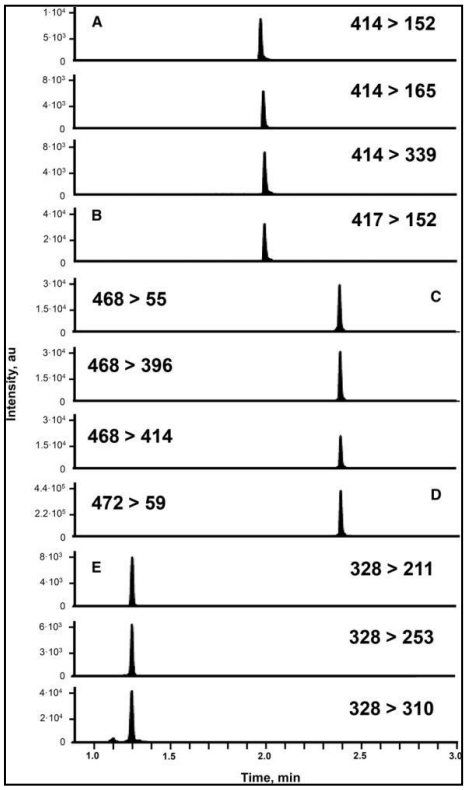

FIGURE 1 . Electrospray ionization + SRM chromatograms for (A) NBUP, (B) NBUP-d3, (C) BUP, (D) BUP-d4, and (E) naloxone. The chromatograms were recorded from a blank head hair sample spiked with BUP at 10 pg/mg, NBUP at 40 pg/mg, naloxone at 50 pg/mg, and IS at 200 pg/mg. Three SRM transitions are reported for the analytes, and 1 transition is shown for the IS.

Compound	LOD (pg/mg)	LOQ (pg/mg)	R^2	Low Level Concentration (pg/mg)	Low Level TR (Bias %)	Low Level IP (CV%)	Low Level ME (Bias %)	High Level Concentration (pg/mg)	High Level TR (Bias %)	High Level IP (CV%)	High Level ME (Bias %)
BUP	0.6	2.2	0.9996	5.0	−4.9	5.5	19.5	500	3.8	2.9	−9.1
NBUP	5.0	17	0.9998	20	1.6	7.8	17.3	2000	−0.9	2.8	−6.9

IP, imprecision; TR, trueness.

TABLE 2 Method Validation: LOD and LOQ Values, Squared Correlation Coefficient, Trueness, Imprecision, and Matrix Effect (ME) (Expressed as Average Percent Bias, n = 5)

Case	Analyzed Hair Length (cm)	BUP Dose (mg/d)	BUP Concentration (pg/mg)	NBUP Concentration (pg/mg)	BUP + NBUP Concentration (pg/mg)	NBUP/BUP Ratio
Patients in maintenance program with constant BUP doses						
1	3.0	1	28	120	148	4.3
2	3.0	2	20	281	301	13.8
3	3.0	2	44	259	303	5.9
4	3.0	2	20	325	345	16.5
5	3.0	2	34	546	580	16.0
6	2.0	2	32	219	251	6.8
7	3.0	3.5	118	1172	1290	9.9
8	3.0	4	51	266	318	5.2
9	3.0	4	33	169	202	5.1
10	3.0	4	60	277	337	4.7
11	3.0	4	46	297	343	6.4
12	3.0	4	49	513	563	10.4
13	3.0	4	57	376	433	6.5
14	3.0	4	126	319	445	2.5
15	3.0	4	95	818	913	8.6
16	3.0	4	117	1008	1126	8.6
17	3.0	5	72	705	777	9.8
18	3.0	6	75	350	425	4.7
19	3.0	6	100	444	544	4.4
20	3.0	6	38	251	289	6.7
21	3.0	6	78	421	499	5.4
22	1.5	6	52	726	778	14.0
23	3.0	7	94	593	686	6.3
24	2.5	8	215	781	996	3.6
25	3.0	8	56	387	443	7.0
26	3.0	8	20	100	120	5.0
27	3.0	9	130	670	800	5.2
28	3.0	15	227	2299	2526	10.1
29	3.0	16	211	1881	2092	8.9
30	2.0	16	122	1684	1806	13.8
31	3.0	16	304	1714	2018	5.6
32	2.5	16	184	789	973	4.3
33	3.0	16	162	361	523	2.2
34	2.5	16	39	732	771	18.6
35	3.0	20	294	2967	3261	10.1
36	3.0	21	395	2197	2592	5.6
37	3.0	32	448	2823	3271	6.3
Patients with irregular BUP daily intake						
38	2.0	2	57	420	477	7.4
39	3.0	2	72	579	651	8.0
40	3.0	4	364	2090	2454	5.7
41	2.5	4	45	231	276	5.2
42	3.0	4	112	619	731	5.5
43	2.5	6	149	1718	1866	11.6
44	3.0	6	10	21	31	2.2
45	1.0	8	33	92	125	2.8
46	3.0	8	74	899	973	12.2
47	3.0	16	49	196	245	4.0
48	3.0	16	80	579	659	7.2
49	3.0	22	48	337	385	7.0

(continued on next page)

TABLE 3 -a The Length of Analyzed Hair Segments, the Oral BUP Intake Assumed During Drug Replacement Therapy, BUP and NBUP Concentrations and Their Summed Values, and the Metabolite-to-Parent Drug Ratio Are Reported

TABLE 3. (*Continued*) The Length of Analyzed Hair Segments, the Oral BUP Intake Assumed During Drug Replacement Therapy, BUP and NBUP Concentrations and Their Summed Values, and the Metabolite-to-Parent Drug Ratio Are Reported

Case	Analyzed Hair Length (cm)	BUP Dose (mg/d)	BUP Concentration (pg/mg)	NBUP Concentration (pg/mg)	BUP + NBUP Concentration (pg/mg)	NBUP/BUP Ratio
50	2.5	24	93	491	584	5.3
51	3.0	24	76	560	636	7.4
52	3.0	32	9	29	38	3.2

TABLE 3 -b The Length of Analyzed Hair Segments, the Oral BUP Intake Assumed During Drug Replacement Therapy, BUP and NBUP Concentrations and Their Summed Values, and the Metabolite-to-Parent Drug Ratio Are Reported

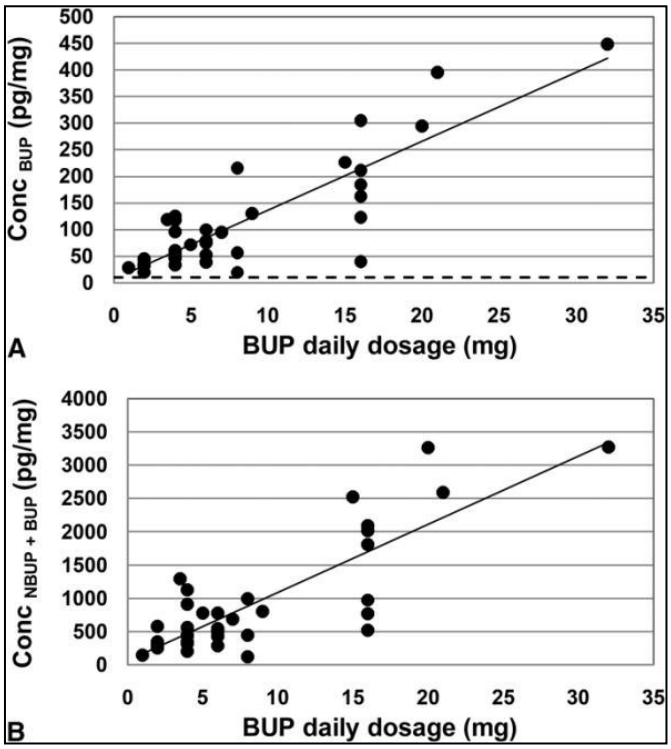

FIGURE 2 . For the interindividual study: (A) dose-concentration relationship between oral daily BUP intake and head hair concentrations for subjects in maintenance replacement therapy. Dotted black line: cutoff value at 10 pg/mg. Interpolating regression line is also shown (Y1 = 12.98x + 6.18). B, Dose-concentration relationship between oral daily BUP intake and summed head hair concentrations of BUP and NBUP. Interpolating regression line is also shown (Y2 = 102.52x + 63.77).

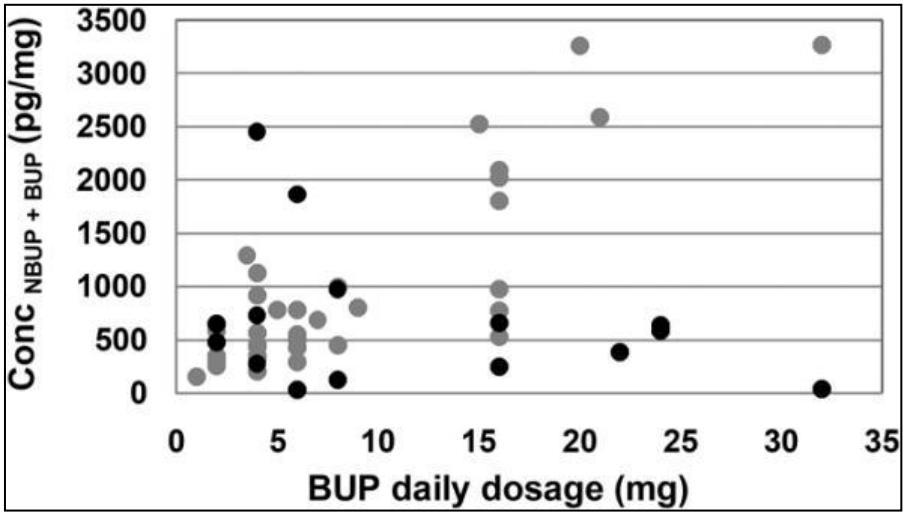

FIGURE 3 . Dose-concentration relationship between oral daily BUP intake and summed head hair concentrations of BUP and NBUP. Black circles: subjects with irregular BUP oral intake (n = 15). Gray circles: subjects in maintenance program (n = 32).

Case	Analyzed Hair Length (cm)	BUP Dose (mg/d)	Difference in Dosage (%)	BUP Concentration (pg/mg)	NBUP Concentration (pg/mg)	BUP + NBUP Concentration (pg/mg)	Difference in Concentration (%)
53	0 → 1	16	−11	366	466	833	−10
	1 → 3	18		375	552	928	
54	0 → 1	14	−13	235	2285	2519	−13
	1 → 3	16		623	2286	2909	
55	0 → 1	24	+50	210	2161	2372	+41
	1 → 3	16		218	1461	1679	
56	0 → 2	2	−50	20	169	190	−38
	2 → 4	4		43	261	304	
57	0 → 3	4	−50	16	233	249	−44
	3 → 6	8		31	411	443	
58	0 → 4	4	+100	31	605	636	+81
	4 → 6	2		20	331	351	
59	0 → 1	4	−33	378	1089	1467	−17
	1 → 3	6		501	1257	1758	
60	0 → 1	16	−11	129	1934	2063	−2
	1 → 2	18		119	1992	2111	
61	0 → 2	8	−33	243	2693	2937	−42
	2 → 3	12		280	4803	5083	
62	0 → 1	8	+100	170	1913	2083	+18
	1 → 3	4		141	1624	1765	
63	0 → 1	5	−38	133	266	399	−7
	1 → 3	8	−33	185	245	430	−18
	3 → 6	12		303	219	522	
64	0 → 1	11	+38	100	1111	1211	+12
	1 → 3	8		99	982	1081	

Differences in oral dosage and summed head hair concentrations through the segments are also reported.

TABLE 4 The Length of the Analyzed Hair Segments, BUP Oral Dosages, BUP, NBUP, and Summed Head Hair Concentrations Are Reported