Int J Legal Med (2011) 125:277–281
DOI 10.1007/s00414-011-0555-8

ORIGINAL ARTICLE

Buprenorphine and norbuprenorphine findings in hair during constant maintenance dosage

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Received: 19 December 2010 / Accepted: 19 January 2011 / Published online: 8 February 2011

Abstract It is still a matter of debate whether a positive correlation between the dose and the amount of drug in the hair exists. Drugs such as buprenorphine (BUP) used under controlled conditions present an opportunity to prove a possible relationship. Due to discrepant findings of BUP/norbuprenorphine (NBUP) ratios in hair, in vitro degradation of both analytes in diluted acid was also investigated. The levels of BUP and NBUP in proximal hair sections from 18 subjects participating in a maintenance program were determined by liquid chromatography/tandem mass spectrometry following incubation with methanol and subsequent liquid/liquid extraction. BUP and NBUP were incubated in diluted hydrochloric acid at 60°C for up to 24 h. The alleged rearrangement products were simultaneously monitored. All hair samples tested positive for BUP (lower limit of detection–0.238 ng/mg hair) and NBUP (0.043–0.961 ng/mg hair). The concentration of NBUP in hair was consistently higher than that of BUP except for a single specimen. Degradation of BUP and NBUP was dependent on time; hydrolysis of NBUP occurred faster than that of BUP. The concentration of BUP and NBUP will be underestimated if analytes are recovered by acidic procedures. NBUP should be monitored in hair samples besides BUP for the sum of both BUP and NBUP may provide an estimate of BUP exposure following long-term administration of the drug.

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Keywords Hair analysis · Buprenorphine · Norbuprenorphine · Acid degradation · Dose–concentration relationship

Introduction

Hair allows a subject's drug use to be traced back through weeks and months, and has become an important matrix for drug analysis in both forensic and clinical settings. The general routes by which drugs can enter the hair are from the bloodstream during the hair growth phase and from the secretions of the sebaceous and sweat glands. From an interpretative point of view, the most important route is via the blood implying that a certain dose–concentration relationship should exist between individuals. Indeed, several studies have shown a positive correlation between dose and amount of drug in the hair, whereas such an association was lacking in other studies [1].

Analysis of drugs in hair such as buprenorphine (BUP) used in maintenance programs under controlled conditions presents an opportunity for verifying whether a relationship between the daily dosage and the individual hair concentration is likely to exist. A first study showing a statistically significant association between the cumulative dose of BUP and its concentration in hair from nine subjects has been published by Goodwin et al. [2]. BUP is metabolized mainly by N-dealkylation via CYP3A4 to norbuprenorphine (NBUP) and then by glucuronidation [3]. Both the parent drug and its N-dealkyl metabolite have been detected in hair; however, there was a discrepancy with regard to BUP/NBUP concentration ratios between studies [2]. Some authors reported higher NBUP concentrations in hair, whereas in some studies BUP was detected in higher concentrations than NBUP. Though the acidic extraction

was not considered to account for the discrepant results [2], this aspect has not been followed up.

To evaluate the ability of hair analysis to enable an association between dosage and hair results, we investigated the relationship between the daily dose of BUP administered to 18 subjects participating in a maintenance program and the respective BUP and NBUP concentrations in hair. In addition, in vitro degradation of BUP in diluted hydrochloric acid (HCl) was compared to that of NBUP.

Materials and methods

The study protocol was approved by the Ethics Committee of the Medical Faculty of Mannheim, Germany, and subjects (seven females, 11 males) provided informed consent prior to collection of a hair sample from the posterior vertex. Hair samples were cut as close to the scalp as possible, the root ends were properly labeled, and specimens were stored in a dry place at ambient temperature protected from light. All subjects received BUP daily by the sublingual route at a constant dose for several months at least; the daily dose and demographic data are summarized in Table 1. The levels of BUP and NBUP in the specimens with a total length of 2–4 cm (n = 4) or the proximal 4-cm section (n = 14) were determined by high pressure liquid chromatography/tandem mass spectrometry (LC–MS/MS) following liquid/liquid extraction after incubation of powdered samples with methanol in an ultrasonic bath.

Table 1 Gender, weight (kg), daily dose (mg), and hair color of subjects participating in the study (n=18)

Subject	Gender (m = male; f = female)	Weight (kg)	Daily dose of BUP (mg)	Hair color
1	m	97	8	Brown
2	m	131	5	Light brown
3	m	105	11	Dark brown
4	f	74	10	Light brown
5	f	72	12	Light brown, bleached
6	m	83	8	Honey blond
7	m	75	3	Light brown
8	m	87	12	Dark brown
9	f	61.5	12	Brown
10	m	88	8	Dark brown
11	f	51	6	Honey blond
12	m	85	8	Brown
13	f	47	12	Light brown
14	f	95	2.2	Light brown, cosmetically treated
15	m	85	4	Light brown
16	m	60	8	Dark brown
17	m	90	8	Light brown
18	f	55	6	Brown

Pure substances (approximately 500 ng each) were incubated in 1 mL 0.1 M HCl at 60°C for up to 24 h to mimic conditions that have been used for processing hair specimens, with aliquots (n=2, each) being analyzed after 0.5, 1, 2, 4, 6, and 24 h to study degradation of BUP and NBUP at an acidic condition.

Materials

BUP (1 mg/mL methanol), NBUP (1 mg/mL methanol), BUP-d_4 (1 mg/mL methanol), and NBUP-d_3 (1 mg/mL methanol) were obtained from LGC (Wesel, Germany). High pressure liquid chromatography grade acetonitrile and methanol (≥99.8%), acetic acid (100%), ammonium acetate (≥98%), 1-chlorobutane, and dichloromethane were purchased from Roth (Karlsruhe, Germany). Double distilled water was from Braun (Melsungen, Germany); HCl and 1 M sodium hydroxide (NaOH) solution was from Merck (Darmstadt, Germany). Drug free, pooled Caucasian hair was obtained from Kerling International (Backnang, Germany).

Preparation and extraction of hair and degraded substances

The hair samples were washed twice with 2 mL dichloromethane 3 min at ambient temperature, allowed to dry, and pulverized in a ball mill (Retsch, Haan, Germany). Following addition of 2 mL of methanol and internal standards (4 ng BUP-d_4 and NBUP-d_3) to approximately 40 mg of pulverized hair, extraction was performed by ultrasonication at 35°C for 4 h and, subsequently, by incubation in a water bath at 40°C overnight. Supernatants were evaporated to dryness, residues were re-dissolved in 500 μL 0.1 M NaOH and 1 mL of 1-chlorobutane/acetonitrile (4:1 v/v) was added. Samples were vortexed (60 s), gently shaken (15 min), and centrifuged (3,000×g, 4°C, 10 min). Nine hundred microliters of the supernatant was dried under nitrogen (40°C) and re-dissolved in 50 μL of the mobile phase prior to analysis.

Samples from the degradation experiment were extracted using 1 mL of 1-chlorobutane/acetonitrile (4:1 v/v) after they had cooled and 1.5 mL 0.1 M NaOH and respective internal standards (200 ng) had been added. A sample of BUP and NBUP (ca. 500 ng, n = 3, respectively) with HCl and NaOH being successively added was directly extracted to yield the initial analytes' concentration.

Instrumentation and MS/MS conditions

Analysis was performed on an API 4000 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) with a TurboIon ionization source operated in the positive-ion mode. The device was interfaced to a HPLC pump equipped with an autosampler (1100 series; Agilent, Waldbronn, Germany). Chromatographic separation was achieved on a Zorbax Eclipse XDB 8 (2.1×150 mm, particle size 5 μm; Agilent, Waldbronn, Germany) with acetonitrile/methanol/4 mM ammonium acetate, pH 3.2 (35:35:30 by volume) as the mobile phase at a flow rate of 250 μL/min. Firstly, the spectra of BUP and NBUP were established by flow injection of the pure compounds. Then, optimized data were acquired in the multiple reaction monitoring mode. The following selected reactions were monitored for quantitation of BUP and NBUP (*m/z*): 468→468 (472→472) for BUP (BUP-d_4); NBUP (-d_3), m/z 414→414 (417→417). The alleged acid rearrangement products of BUP and NBUP were monitored at (*m/z*): 436→436, 382→382.

Evaluation of the analytical method

A calibration curve was obtained by analyzing powdered drug-free hair (40 mg) enriched with BUP and NBUP at 0.02, 0.05, 0.1, 0.5, 0.75, and 1.0 ng/mg and internal standards (4 ng BUP-d_4 and NBUP-d_3, respectively); a blank hair (without analyte and internal standard) and a zero sample (containing the internal standard only) were also prepared for each run. A calibration curve for the specimens of the degradation study was generated from 1.0 mL 0.1 M HCl to 1.5 mL 0.1 M NaOH fortified with BUP and NBUP (100, 300, 500, and 1,000 ng, respectively) and respective internal standards (200 ng each).

Ion suppression was checked by comparing a set of BUP and NBUP solutions (4 and 30 ng/50 μL each, $n = 3$, mobile phase) to an additional set of 40 mg blank hair samples extracted with analytes (0.1 ng/mg, 0.75 ng/mg, $n = 3$, respectively) added just before injection into the liquid chromatograph.

Analyte concentrations were determined from the peak area of the respective analyte to the peak area of the corresponding internal standard, and ratio comparison to the calibration line (Analyst 1.1.4 software; Applied Biosystems). The lower limits of detection (LLOD) and of quantification (LLOQ) were estimated from the respective calibration curve [4].

Extraction efficiency was determined by comparing the peak area ratios calculated when BUP, NBUP, and their deuterated analogues were extracted together ($n = 5$, 0.1, and 0.75 ng/mg hair) to the peak area ratios obtained when BUP and NBUP were extracted ($n = 5$, 0.1, and 0.75 ng/mg hair), and deuterated compounds were added just before reconstitution.

Imprecision data were assessed using fortified blank hair ($n=5$, 0.1, and 0.75 ng/mg hair) and expressed as the percent coefficient of variation about the mean values.

Results

The retention time was 3.15 min for BUP and 2.01 min for NBUP. Calibration curves were linear through the entire concentration range for both BUP and NBUP ($r > 0.998$). LLODs/LLOQs were estimated at 0.003/0.008 and 0.004/0.010 ng/mg hair for BUP and NBUP, respectively. Recovery rates of BUP and NBUP from hair was within the range of 80–90% to 43–47% at the low and high concentration chosen. Ion suppression was not observed by MS/MS down to the respective LLOQs. The coefficient of variation expressed as a percentage did not exceed 5.7% in imprecision measurements.

All hair specimen tested positive for BUP (mean = 0.069 ng/mg hair, range = LLOD–0.238 ng/mg hair) and NBUP (mean = 0.233 ng/mg hair, range = 0.043–0.961 ng/mg hair). NBUP concentrations were always higher than those of BUP except for sample 1 (Fig. 1); however, the molar concentration ratio of parent drug and metabolite was highly variable (mean = 0.34, range =0.015–1.19).

Incubation of BUP and NBUP at an acidic condition revealed both compounds being degraded dependent on time; acidic hydrolysis of NBUP occurred faster than that of BUP (Fig. 2). In addition, peaks that increased with increasing incubation time could be detected at the transitions of the alleged acid rearrangement products of the respective analytes. There was no evidence that such products will be formed using the present specimen preparation.

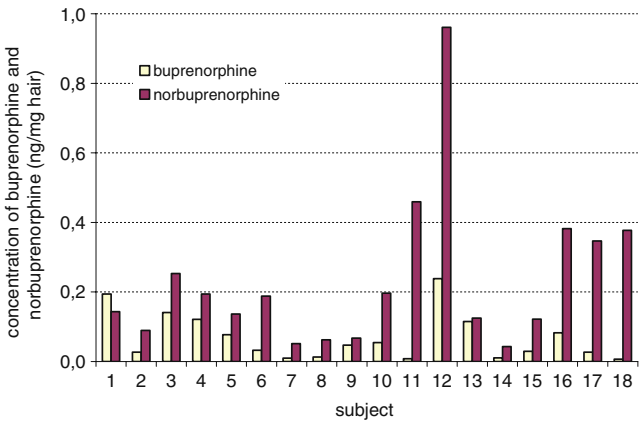

Fig. 1 Concentrations of buprenorphine and norbuprenorphine (ng/mg hair) in proximal hair segments (≤4 cm) of subjects at long-term medication of BUP

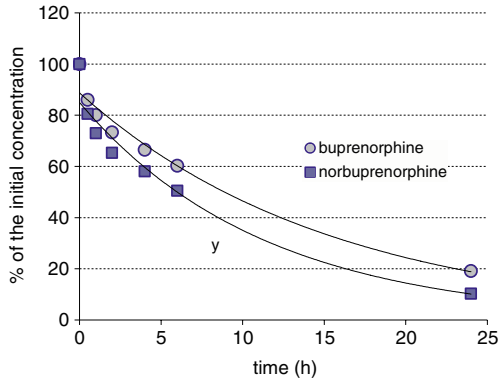

Fig. 2 Decrease of BUP and NBUP (500 ng each, expressed as a percentage of the initial concentration, %) with increasing incubation time at 60°C in 1.0 mL 0.1 M HCl

The absolute dose of BUP ranged from 2.2 to 12 mg per day, and varied from 0.02 to 0.26 mg BUP/kg weight. A linear relationship ($y = 6.101x - 0.203$, $r = 0.851$, Fig. 3) could be established between the weight-related dose depending on the concentration of both BUP and NBUP in hair samples—with NBUP referred to the molecular weight of BUP—if results <LLOQ ($n = 1$) and from cosmetically treated hair ($n = 2$) were excluded from final data analysis.

Discussion

The present investigation focuses on the ratio of BUP/NBUP, and on a possible relationship between the daily dose of BUP and the amount of BUP and its major metabolite, NBUP, determined from proximal hair sections of subjects undergoing BUP maintenance treatment.

BUP and NBUP could be identified in all specimens with NBUP being incorporated into hair in comparatively higher amounts than BUP with the exception of sample 1. This finding is consistent with the results reported by Goodwin et al. [2] and Wilkins et al. [5]. At the same time, it is a remarkable finding in view of the fact that a higher concentration of the parent drug compared to that of its dealkylated metabolite is mostly detectable in hair, such as e.g. for doxepine [6] or ketamine [7]. Nonetheless, the observation that the mean steady-state concentration of NBUP in blood often exceeds the concentration of BUP following chronic sublingual administration in humans may account for the present findings [3, 8]. Not only the elimination half-life of the metabolite is longer than that of the parent drug but also its rate of elimination has been shown to decrease with increasing blood concentration [9]. However, caution is advisable extrapolating the present data to a single or occasional use of BUP.

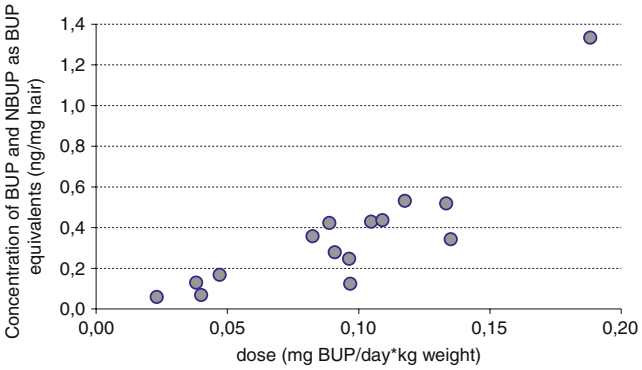

Fig. 3 Relationship (y=6.101−0.203, r=0.851) between the hair assay result (sum of BUP, and NBUP, expressed as BUP equivalents) and the daily dose referred to the individual's body weight (n=15)

BUP concentration measured from hair specimens of opiate-dependent subjects maintained with BUP typically range from 0.040 to 2.360 ng/mg hair [10], which covers the results obtained from the present investigation. A high degree of inter subject variability in measured hair concentrations of BUP can be observed even when subjects received the same dose which may be due to the extensive polymorphism occurring in CYP3A4 of the Caucasian population [11].

Degradation of NBUP upon treatment with diluted acid occurred faster than that of BUP, which may partly explain the different results following alkaline digestion or acidic extraction of hair from individuals treated with BUP. BUP and NBUP undergo an acid-catalyzed rearrangement reaction resulting in a loss of methanol and ring formation between the 6-methoxy group and the branched side chain (Fig. 4); the stable reaction products have been proposed to improve quantitative properties for gas chromatographic/mass spectrometric analysis of BUP and NBUP [12]. A diminished recovery of NBUP from hair has been observed following acid digestion [2]. Even negative NBUP findings along with positive BUP findings in hair at an LOD of 0.01 ng/mg hair after an overnight incubation in diluted acid have been reported [13]. Also, BUP was found in more abundant quantity compared to NBUP following incubation of hair at 56°C overnight in 1 mL 0.1 M HCl [13–16]. An acidic extraction procedure has been considered unlikely to account for the discrepant results as Vincent et al. [17]

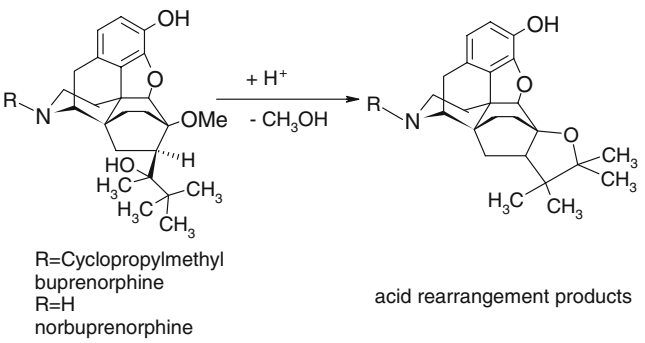

Fig. 4 Acid rearrangement products of buprenorphine and norbuprenorphine following incubation in diluted acid (modified according to reference [12])

reported higher NBUP concentrations in four out of five hair specimens following incubation in diluted acid. However, hair specimens and drug use were not exactly specified, whereas the present investigation focused on proximal sections including only subjects on long-term treatment with known doses of BUP. It may be speculated whether NBUP may be less tightly bound to hair compared to BUP, and may increasingly be lost following weathering of distal hair sections. The present results clearly indicate that degradation occurs rapidly, and that the concentration of both BUP and NBUP in hair will be underestimated if analytes are recovered by acidic procedures.

Nowadays, drugs in hair can reliably be detected using an appropriate assay. Whether hair analysis can effectively indicate an individual's drug intake is still a matter of debate. A part of this controversy is related to the accuracy of self-reported drug use and the routes by which drugs can enter the hair. The simplest model proposed is by passive diffusion of drug from the bloodstream into the growing hair suggesting incorporation to be dependent on drug concentration in blood which in turn is dependent on the ingested dose. Today, a more complex model has been accepted suggesting drugs to be incorporated in addition to blood via sweat and sebum or from body stores [1]. The transfer of BUP into sweat has successfully been tested [2]; nevertheless, further evidence is needed to clarify whether BUP present in sweat or being transferred from body stores contributes to drug deposition into hair. The present results suggest blood circulation as a major source of BUP and NBUP to enter the hair.

An increase in the concentrations of both BUP and NBUP with increasing dose was evident. This is in line with findings on methadone and cannabinoids in hair following long-term exposure [18, 19]. Also, a reasonable positive relationship could be reached between the hair assay result and the daily dose referred to the individual's body weight if the sum of BUP and NBUP concentrations was considered. An approximate dose-dependent increase of BUP/NBUP concentrations in pigmented hair could be established in a rat model [5]. BUP being a cation at physiologic pH (pK_a = 8.5, 10.0), the drug is expected to be incorporated into pigmented hair to a greater extent than into non-pigmented hair. Using cosmetically non-treated blond to dark brown hair specimens for data analysis in the present study, the finding of a dose-related incorporation appears not quite unexpected.

Hence, determination of NBUP in hair should not only be performed by an appropriate assay for further confirmation of BUP administration; considering both the parent drug and its major metabolite in pigmented hair may allow a gross estimate of the degree of BUP exposure following long-term administration of BUP.

Conflict of interest The authors declare that they have no conflict of interest.

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