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Dosage de la buprénorphine et de la norbuprénorphine dans les cheveux par GC-MS

Determination of buprenorphine and norbuprenorphine in hair by GC-MS

Critiana STRAMESI*, Alessandra ZUCCHELLA, Claudia VIGNALI, Lucia POLITI, Angelo GROPPI, Aldo POLETTINI

Department of Legal Medicine & Public Health, Unerversity of Pavia, Via Forlanini 12 - 27100 PAVIA - Italy

*Corresponding Author: Cristiana STRAMESI, Department of Legal Medicine & Public Health, University of Pavia, Via Forlanini 12 - 27100 PAVIA - Italy
Tél : +390382987809 - Fax : +39038258025 - E-mail : stramesi@unipv.it

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RÉSUMÉ

La buprénorphine est un dérivé synthétique de la thébaïne utilisée comme produit de substitution de l'héroïne dans les programmes de désintoxication. La buprénorphine a été présentée pour cette application en Italie en mai 2000 et son utilisation pour cette thérapeutique a augmenté de plus de 30% dans les 3 dernières années. Le but de cette étude a été d'étendre la méthode appliquée en routine dans notre laboratoire à la détection des métabolites de l'héroïne, de la cocaïne et des amphétamines ainsi qu'à la buprénorphine (BPR) et son métabolite la norbuprénorphine (norBPR) afin de surveiller l'administration de BPR dans les programmes de désintoxication et de détecter une éventuelle toxicomanie pendant la désintoxication. La procédure analytique est la suivante : après une étape de lavage dans le méthanol, les cheveux ont été finement coupés et incubés dans du HCl 0,1N (45°C, pendant la nuit). La nalorphine a été choisie comme standard interne. La purification des stupéfiants a été faite par une extraction en phase solide (Bond Elut Certify®) qui a un taux de récupération supérieur à 80% pour la BPR et la norBPR ; l'extrait purifié a été dérivé par le N-méthyl, N-triméthylsilyl trifluoroacétamide (MSTFA) et l'analyse a été

SUMMARY

Buprenorphine is a synthetic derivative of thebaine used as a substitute of heroin in detoxification programs. Buprenorphine was introduced for this application in May 2000 in Italy and its use for this therapeutic purpose has increased by over than 30% in three years. The aim of this study was to extend the method routinely applied in our laboratory to detect heroin metabolites, cocaine and amphetamines also to buprenorphine (BPR) and its metabolite norbuprenorphine (norBPR) in order to monitor BPR administration in detoxification programs as well as to detect drug abuse during detoxification. The analytical procedure was as follows: after a washing-step with methanol, hair was finely cut and incubated in HCl 0.1N (45°C, overnight). Nalorphine was chosen as internal standard. Purification of analytes was executed by solid phase extraction (Bond Elut Certify®) which provided recoveries higher than 80% for both BPR and norBPR; purified extract was derivatised with N-methyl, N-trimethylsilyl trifluoroacetamide (MSTFA) and the analysis was performed by GC-MS in SIM mode. Ions monitored were: m/z 450, 482, 506 for BPR, m/z 468, 500, 524 for norBPR and m/z 455, 414, 324 for internal standard

conduite sur une GC-MS en mode SIM. Les ions suivis étaient : m/z 450, 482, 506 pour la BPR, m/z 468, 500, 524 pour la norBPR et m/z 455, 414, 324 pour le standard interne (les ions soulignés ont été utilisés comme quantificateurs). La validation de la méthode a été réalisée par : l'évaluation de l'exactitude et de la précision ; l'analyse de sept échantillons de cheveux sains ; le test de linéarité (0-0,5 ng/mg, n=5). Les précisions intra-jour (n=7) et inter-jour (n=3 sur 5 jours différents) étaient supérieures à 8,8% pour les deux stupéfiants et l'exactitude supérieure à 15%. La limite de détection était de 0,005 ng/mg et la limite de quantification de 0,01 ng/mg. Cette méthode a été appliquée sur des échantillons de cheveux prélevés sur des patients ayant pris part à des programmes de désintoxication. Elle a prouvé sa facilité d'application en routine.

(underlined ions were used as quantifiers). Method validation was performed by: evaluation of accuracy and precision; analysis of seven drug-free hair samples; testing of linearity (0-0.5 ng/mg, n=5). Intra-day (n=7) and inter-day (n=3 on 5 different days) precision were better than 8.8% for both analytes and accuracy better than 15%. The limit of detection was 0.005 ng/mg and the limit of quantitation was 0.01 ng/mg. This method was applied to hair samples collected from patients in withdrawal treatment programmes and demonstrated its good applicability in routine analysis.

Introduction

Buprenorphine (BPR) is a semisyntethic opioid derivative, obtained from thebaine, with mixed agonist/antagonist activity. This drug is a partial mu receptor agonist and a kappa receptor antagonist and it has got two main applications: as a narcotic analgesic (Temgesic®) and as an opioid substitute (Subutex®, available at 2 and 8 mg dosages).

After oral administration, activity appears after 20 minutes, and the peak plasma concentration is observed about two hours afterwards. Effects are present until up to 72 hours after administration.

Its main metabolite is desalkyl-buprenorphine or norbuprenorphine (nor BPR) and both drugs are glucurono-conjugated.

BPR was first used in substitutive therapy in France in 1996. Currently, Buprenorphine Substitutive Treatment (BST) is available in 28 countries - 20 EU member states -. and has been approved, though not yet launched, in 7 further countries. According to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), BST has been increasing over the last years (30%). Subutex is available in tablets for sublingual use.

In Italy, BPR was introduced as medication for the substitutive management of opiates-dependent individuals in May 2000. BPR therapy is delivered by public drug treatment units (Ser.T.) which are part of the national health system (NHS). It is usually dispensed every two days and the patient compliance is monitored via urine analysis. Weekly doses are dispensed for home administration only after three months or over of opiate-negative urines samples.

Owing to the expected spread of BPR use and abuse, the inclusion of BPR and norBPR in routine urine and hair analysis has become necessary.

Few analytical methods for the determination of BPR in hair have been published so far. Recently, Cirimele (2) et al. have developed an ELISA screening for BPR in hair followed by confirmation of positives by LC-MS (3). Other laboratories perform the analyses using GC-MS after derivatisation with trimethyl-silyl donors (4) or LC-MS-MS (5). Overnight incubation of hair in diluted HCl is usually preferred, except for Wilkins and colleagues (5) who digested hair in soda. Extraction is carried out either by solvent partition (3, 5) or SPE (4).

The aim of this study was to extend the GC-MS method routinely applied in our laboratory to detect heroin metabolites, methadone, cocaine and amphetamines (1) also to BPR and its metabolite norBPR in order to monitor BPR administration in detoxification programs as well as to detect BPR abuse. This would allow to widen the profile of drugs of abuse detected avoiding collecting additional sample and saving time and money.

Experimental

Chemicals and reagents

Pure standards of buprenorphine (BPR), norbuprenorphine (norBPR), nalorphine were purchased from SALARS (Como, Italy). *N*-Methyl, *N*-trimethylsilyl trifluoroacetamide (MSTFA) was purchased from SIGMA. Methanol, dicloromethane, propan-2-ol, hydrochloric acid and ammonium hydroxide (Fluka Biochemika, Switzerland) were reagent grade. Bond Elut Certify® LCR cartridges (10 mL capacity, 130 mg) were obtained from Varian (Harbor City, CA, USA).

Hair samples

Drug-free hair for method development, was obtained from laboratory staff volunteers. Positive hair samples (n=9) were collected from patients who had undergone at least three months of maintenance treatment pro-

gramme with buprenorphine.

Sample preparation

Hair samples were processed using the method routinely applied in our laboratory for drugs of abuse. All samples were decontaminated with methanol (1 mL, vortex, centrifuge 4500 rpm, 5 min); after drying, hair was finely cut into segments of 1 mm or less. The hair sample (50 mg) was then spiked with the internal standard (nalorphine) and incubated with HCl 0.1 M overnight at 45 °C. Samples were cooled at room temperature and the pH was adjusted to 6 using NaOH 2 M and phosphate buffer 0.1 M, pH 6.0 (1 mL). The incubation media were then purified by solid phase extraction with Bond Elut Certify® cartridges: cartridges were conditioned with methanol (2 mL) and phosphate buffer 0.1 M, pH 6 (2 mL) and, after sample percolation, rinsed with water (2 mL), HCl 0.1 M (3 mL), and methanol (5 mL). Analytes were eluted with a mixture of dichloromethane:propan-2-ol (8:2) containing 2% ammonium hydroxide (1mL + 1mL). The organic phase was evaporated to dryness under nitrogen flow and derivatised with 50 µl of MSTFA (75 °C, 15 min).

GC-MS analysis

Hair extracts were analysed using a Hewlett-Packard (Palo Alto, CA, USA) 6890 gas chromatograph equipped with a 5973 mass detector and a 7673 automatic injector. Separation (pulsed splitless, 1 min with a column head pressure of 172 kPa) was carried out using a HP ultra 2 (5% phenil, methylsilicone) fused-silica capillary column (12 m x 0.2 mm i.d., 0.33 µm film thickness). Helium was used as a carrier gas at a flow rate of 1 mL/min (costant flow mode). The operative temperatures were as follows: injector: 280 °C; column: from 150 °C(1.5 min) to 300 °C at 50 °C/min, final isotherm for 14.50 min; transfer line: 280 °C. The mass spectrometer was operated in selected ion monitoring (SIM) acquisition mode. Ions monitored were: *m/z* 450, 482, 506 for BPR; *m/z* 468, 500, 524 for nor BPR; *m/z* 455, 414, 324 for internal standard (nalorphine). Underlined ions were used for the quantification.

Method validation

The following criteria were used for validation: specificity, sensitivity, linearity, intra and inter-day precision and accuracy, recovery.

The specificity of the method was assessed by checking for the presence of interfering substances at the retention time of BPR, norBPR and internal standard in seven different hair samples collected from non users.

Linearity was investigated by least squares regression model and expressed by the correlation coefficient (R^2). 5-point calibration curves with calibrators at 0, 0.02, 0.05, 0.1, 0.2, 0.5 ng/mg (5 replicates for each point) of BPR and norBPR were prepared.

The sensitivity of the method was evaluated by determining the limit of detection (LOD) and the lower limit of quantification (LLOQ). The LOD was defined as the concentration with a signal-to-noise ratio of at least 3, and the LLOQ as the lowest concentration with a signal-to-noise ratio at least 10.

Precision was expressed as the relative standard deviation of the control sample concentration calculated using the calibration curve, and accuracy was expressed as the relative error of these concentrations. The validation protocol included intra- and interday analyses. The intraday assay (0.05, 0.2, 0.5 ng/mg; n=7) was carried out by 3 different operators during the same day. Interday analyses (0.05 - 0.2 - 0.5 n=3) were performed using five assays, which were carried out over four consecutive days by different operators; the fifth assay, after correction of pH, was kept at 4 °C and analyzed after the weekend .

Extraction recoveries of analytes were calculated by comparison of the peak area ratios obtained after analysis of spiked samples with the mean value of those obtained when the standards were added to extracted blank hair samples (representing 100% of extraction recovery).

Results and discussion

After the analysis of seven blank hair samples, no interferences were detected at the retention times of BPR, norBPR and internal standard (figure 1).

Linearity correlation was found for both substances in the range 0-0.5 ng/mg; the slopes, intercepts and the average linear correlation coefficients (R^2) are presented in table I. Calibration curves are expressed as $y = ax + b$ where x is the concentration of analyte and y is the ratio between peak area of analyte and peak area of internal standard.

The LOD and the LLOQ were calculated at 0.005 ng/mg and 0.01 ng/mg respectively. These LOD and LLOQ well agree with concentrations measured in real hair samples of BST patients/BPR abusers as reported in the literature.

Results of intra-day and inter-day precision and accuracy are shown in table II and III. Intra-day and inter-day precision was better than 8.8% for both analytes and accuracy better than 15%.

Solid phase extraction provided recoveries higher than 80% both for buprenorphine and norbuprenorphine.

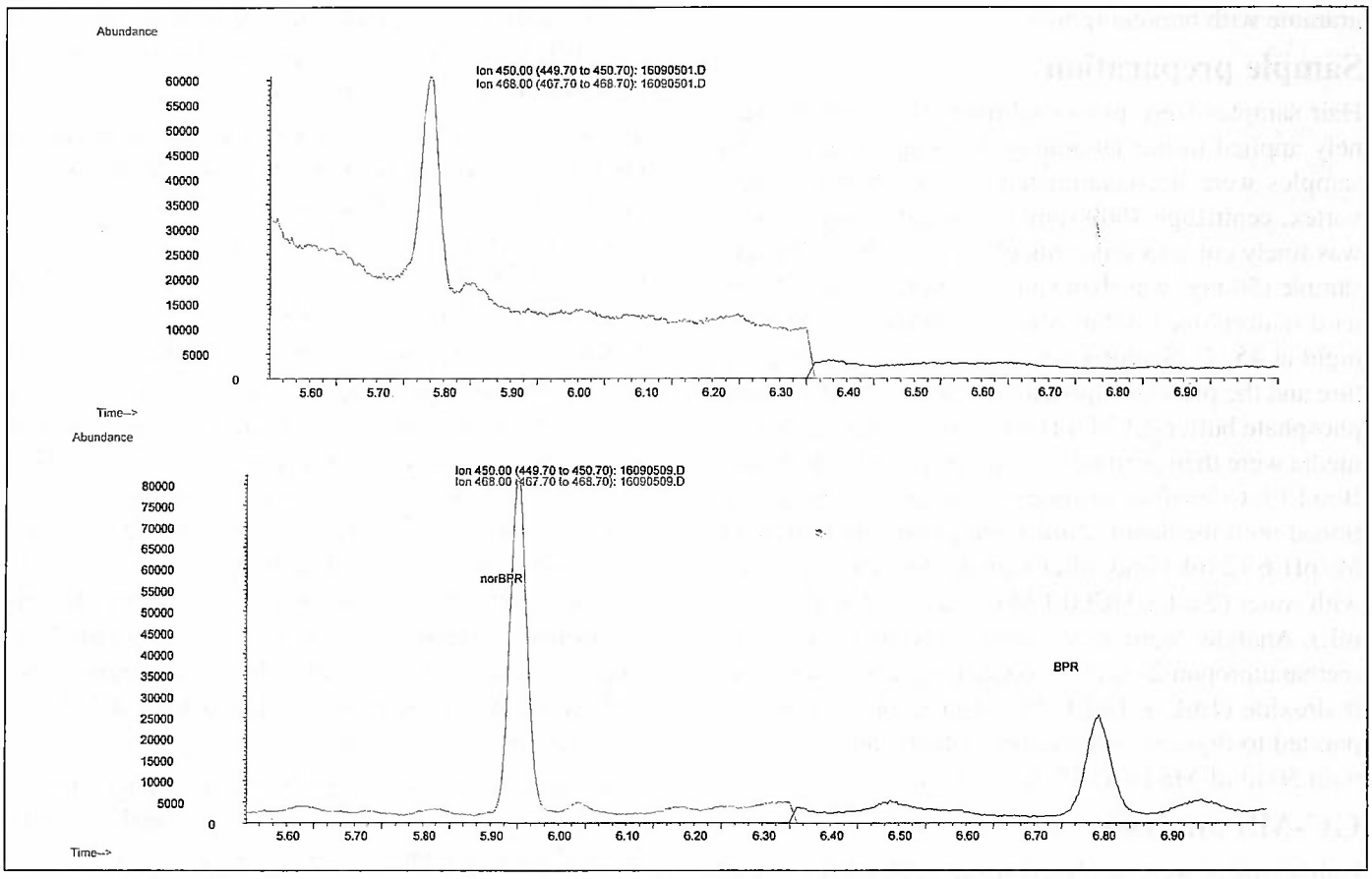

Figure 1 : *Selected Ion Chromatogram of Buprenorphine and norBuprenorphine in a blank hair and in a real sample.*

Table I : *Characteristics of calibration curves.*

Compound	Concentration range	No. of curves	Slope*	Intercept*	Correlation coefficient (R^2)*
Buprenorphine	0 - 0.5 ng/mg	5	1.6385 ± 0.1	0.028 ± 0.009	0.994 ± 0.005
norbuprenorphine	0 - 0.5 ng/mg	5	1.5492 ± 0.2	0.025 ± 0.01	0.990 ± 0.003

*mean ± SR

Table II : *Intraday and interday precision.*

Intraday precision	Coefficient of variation (%)	
	BPR	NorBPR
0.05 ng/mg	7.76	8.34
0.2 ng/mg	6.45	8.82
0.5 ng/mg	4.58	5.62
Interday precision		
0.05 ng/mg	8.03	8.79
0.2 ng/mg	7.99	8.52
0.5 ng/mg	6.52	5.77

Table III : *Intraday and interday accuracy.*

Intraday accuracy	Accuracy (%)	
	BPR	NorBPR
0.05 ng/mg	14.9	13.5
0.2 ng/mg	2.5	5.14
0.5 ng/mg	3.3	10.6
Interday accuracy		
0.05 ng/mg	11.7	13.16
0.2 ng/mg	9.12	3.7
0.5 ng/mg	4.7	12.2

Real samples

9 hair samples collected from one female and eight male patients under BPR maintenance treatment at different dosages (2-16 mg/die) were examined. Analysis was performed on 3-cm proximal segments and, when available, on the adjacent 3-cm segment (4 samples). All samples tested positive for BPR, and norBPR was present in 12 out of 13 (table IV). BPR concentrations ranged from 0.011 to 0.105 ng/mg, norbBPR levels were between not detected and 0.528 ng/mg. BPR/norBPR ratio was found to be lower than one in all but one sample.

Real samples concentration measured are in agreement with the ones reported in literature for BST patients/BPR abusers, as listed in table V.

A correlation between BPR daily dosage and hair concentration can be roughly observed. At a dosage range of 2 - 6 mg/die the total buprenorphine concentration (BPR + nor BPR expressed as BPR equivalents) was 0.22 ± 0.04 while for dosages of 8-16 mg/die it

Table IV : *Concentrations of real samples.*

Hair Sample	BPR ng/mg	NorBPR ng/mg
1a	0.043	0.220
1b	0.038	0.127
2a	0.105	0.121
2b	0.044	0.149
3	0.020	0.131
4	0.070	0.123
5	0.011	n.d.
6	0.014	0.013
7a	0.023	0.236
7b	0.013	0.187
8	0.026	0.528
9a	0.015	0.261
9b	0.015	0.365

* a, b indicate proximal (0-3 cm) and distal (3-6 cm) segments, respectively, n.d. not detected

was 0.31 ± 0.19. When the distal (3-6 cm) hair segment was available, the total BPR concentration was always lower than in the proximal (0-3 cm) segment. This finding could be explained with partial removal of the analytes from hair in the distal segment where the cuticle may be damaged.

The ratio BPR/norBPR tends to decrease at higher doses, possibly due to induction of BPR metabolism at higher doses.

Conclusion

This study demonstrates that the method routinely used in our lab for the analysis of drugs of abuse in hair can be extended to BPR and norBPR without the need of collecting additional sample. In particular, it was proved that the same sample preparation used for heroin metabolites, methadone, cocaine and metabolites, amphetamines and 3,4-methylenedioxyamphetamines provides sufficient recoveries and clean extracts also for BPR and norBPR. However, though the other analytes can be determined within a single chromatographic run, BPR and norBPR detection requires a further injection into the chromatographic system owing to the higher MW of these analytes.

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Table V : *Concentration range of buprenorphine and norbuprenorphine in real hair samples.*

Reference	sample	range	
2	Detoxification treatment, Autopsy (n = 25)	BPR	0.04-0.3 ng/mg
3	Detoxification treatment (n = 6)	BPR	0.004-0.14 ng/mg
		NorBPR	n.d.-0.067 ng/mg
4	Detoxification treatment (n = 5)	BPR	0.06-0.36 ng/mg
		NorBPR	0.03-0.78 ng/mg
6	Drug addicts, overdose, Detoxification treatment (n = 71)	BPR	0.02-8.16 ng/mg
		NorBPR	n.d.-1.47 ng/mg
7	Autopsy (n = 26)	BPR	0.01-1.08 ng/mg
		NorBPR	n.d.-1.02 ng/mg
This study	Detoxification treatment (n = 10)	BPR	0.011-0.105 ng/mg
		NorBPR	n.d.-0.528 ng/mg

n.d., not detected