In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994

Makoto UEKI, Makoto Fujisaki, Shinsuke Miyamoto, Ayako Takahashi, Tomomi Hiruma

Problem in the detection of High Potency Drug (Screening and conclusive confirmation of Buprenorphine)

Doping Control Laboratory, Mitsubishi Yuka Bio-Clinical Laboratories, Inc., Tokyo, JAPAN

Introduction

Buprenorphine,(6R,7R,14S)-17-cyclopropylmethyl-7,8-dihydro-7-{(1S)-1-hydroxy-1,2,2-trimethylpropyl}-6-O-methyl-6,14-ethano-17-normorphine (CAS No.52485-79-7), is a high potency synthetic narcotic drug sold on a market in several countries, e.g. *Lepetane* (Japan), *Buprenex* (USA), *Buprex* (UK, Spain), *Temgesic* (UK, FRG, Norway) etc..
Due to the narcotic agonist-antagonist property of buprenorphine, the drug becomingly used not only as pain killer but also for the treatment and detoxication of opiate dependent addict, however, long term abuse of the drug may be caused to form the dependency.
Two forms of buprenorphine, an injectable form and a suppository, are available in Japan without narcotic control law regulation, furthermore, a project to develop a buprenorphine retard tablet is ongoing.
Problems in the detection of buprenorphine are stem from its acid labile property, low pharmacological dose, extensive metabolism into norbuprenorphine and low excretion of the parent compound into urine.

This paper describes an integrated routine screening procedure of buprenorphine as the main metabolite norbuprenorphine and the conclusive quantification analysis of buprenorphine in urine.

Experimental

Materials

Buprenorphine hydrochloride (BUP), Norbuprenorphine (NBP) and N-cyclobutylmethyl norbuprenorphine (CMBP) as an internal standard were synthesized by our co-workers in Nikken Kagaku Co. and were particularly used for the clinical trial study. Heptafluorobuthylyl anhidride (HFBA), MSHFB and MBHFB were GC grade. The other reagent used were analytical grade or HPLC grade. XAD-2 was obtained from Rohm & Hass and Sep-Pak C_{18} cartridge was purchased from Millipore Waters. Fused silica capillary column was methylsilicone Ultra-1, 0.2mm I.D. x 12.5m, 0.11 μm, from Hewlett Packard.

Excretion study

Urine samples were collected from a healthy male volunteer before and after single oral administration of 2 mg of BUP.

Screening Methods

Urine specimens were analysed according to our known routine screening procedures approved by the Medical Commission of the International Olympic Committee (MC/IOC). NBP could be detected only by the screening procedure-4 in free steroid fraction as the main metabolite NBP.
Residue of urine sample was removed by sedimentation prior to the solid phase extraction. Five ml of urine samples fortified with 50ng of stanozolol as an internal standard were applied onto XAD-2 column (5mm I.D. x 25mm L in pasteur pipette) or Sep-Pak C_{18} and

washed with an equal volume of water. Extraction was done by 5 portions of 0.5 ml methanol and solvent was removed under a stream of nitrogen. The residue was dissolved in 1ml of 0.2M-sodium phosphate buffer pH=7.0 and free steroids were isolated with 5ml of diethylether.

Selective derivatization conditions

Add 30 μl of MSHFBA reagent
(MSHFBA/TMCS/Imidazol=2000 μl/100 μl/40 mg)
Heated at 80°C for 5 minutes. O-Trimethylsilylation
Add 7 μg of MBHFBA
Heated at 80°C for 15 minutes. N-Heptafluorobuthylation

Chromatographic conditions

Instrument	: HP5970 MSD or HP5989 MS Engine equipped with HP7673A ALS		
Column	: Ultra 1 (HP) 0.2mm I.D. X 12.5m L Film thickness 0.11 μm		
Oven temp	: Initial	180°C	hold 0.5 min.
	Rate	30°C/min.	
	Final	290°C	hold 5 min.
Injector	: Temp	290°C	Purge off 30 sec.
	Split ratio 11:1		
Carrier flow	: 70 kpa He at 180°C		
MS conditions	: Ion source	200°C	transfer line 300°C
	Analyzer	250°C	
Workstation	: HP9000-300 Pascal Chemstation with ChemLAN		

Quantification Procedure

One ml of real urine aliquot or a reagent water for blank sample was placed in a silicone coated glassware test tube. For calibration samples, the known amount of BUP in methanol was dried in a silicone coated glassware test tube and the residue was re-dissolved into 1ml of drug free urine. Five μg of CMBP was then added to each analyte as an internal standard (IS). The water layer was adjusted to pH=9.4 by adding of saturated sodium hydrogen sulfate and it was followed by the extraction with 4ml of Ethylacetate / n-Heptane (4/1). BUP was then back extracted into 1ml of 0.05N sulfuric acid. Re-extraction of BUP was done with 4ml of Ethylacetate / n-Heptane (4/1) under alkalinized pH (pH=9.4).
Dry residue of the organic extract was analysed by GC/MS after HFB derivatization.

HFB Derivatization Condition

Dissolve dry residue in 100 μl of ethyacetate.
Add 50 μl of HFBA.
Stand for 20 minutes under room temperature 20±5°C.
Remove solvent under nitrogen stream.
Re-dissolve dry residue in 30 μl of ethylacetate.

5 μl of analyte was injected onto GC/MS column using solid injector (solventless injector).

Quantification conditions

In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994

Instrument	: JMS DX-303 double focusing GC/MS equipped with JMS-DA5000 data station.		
Column	: Ultra 2 (HP) 0.32mm I.D. X 8.0m L Film thickness 0.53 μm		
Carrier	: He		
Oven temp	: Initial	220°C.	
	Rate	32°C/min.	
	Final	330°C.	
Injector	: Temp	295°C.	
Carrier flow	: 70 kpa He at 180°C.		
MS conditions	: Ion source	295°C.	transfer line 295°C
	Ionization	70eV.	
	Ion current	300μA.	
	Ion multiplier	2.4kV.	

Results and Discussion

Integration of Buprenorphine Detection into Screening Procedure

Insufficient sensitivity for the detection of BUP was obtained under routine screening procedure. NBP was only BUP related compound found by our screening procedure-4 in the free steroid fraction (Figure-1). Relative retention time of NBP-O-TMS-N-HFB to that of Stanozolol-O-TMS-N-HFB was 1.100 and recommended monitoring ions (m/z) for SIM analysis of NBP derivative were 624, 606 and 592 (Figure-2 and Table-1). Cyclized artifact of BUP and NBP could be detected by the screening procedure-2 for heavy volatile dope agents with selective derivatization if the sample contained enough amount of the both compounds e.g. 10 μg/ml., however, no such compounds were found in the urine sample of excretion study.

Quantification

BUP can be quantified by any of positive EI, Positive CI or negative CI detection. The best signal to noise ratio for real sample analysis was obtained by SIM analysis with electro ionization detection.

Optimized assay procedure allowed the high sensitive quantification of BUP and NBP. Extraction recovery of BUP, NBP and CMPB was around 75%, 70% and 70% respectively. The LOD, LOQ and the method linearity for BUP and NBP were 50, 100 and up to 10,000 pg/ml. The accuracy and the precision of BUP and NBP were |deviation|=4.4-14.4% and CV=6.1-7.9% for BUP and |deviation|=5.6-13.5% and CV=6.1-8.0% for NBP respectively.

Figure-3 and -4 shows the mass fragmentgram of blank sample and real urine sample collected from a normal adult male at 9 hours after 2mg oral administration of NIK-264 (buprenorphine hydrochloride from Nikken Kagaku Co.).

The figure demonstrating that parent BUP is present in the urine sample and NBP is detected as more intense signal.

Conclusion

Buprenorphine detection procedure was integrated into presented routine screening procedure without any modification of the testing system. An optimized procedure for the quantification of BUP and NBP was also discussed.

References

1) Pharmacokinetics of Morphine and Its Surrogates X: Analyses and Pharmacokinetics of Buprenorphine in Dogs., Edward R. et. al., Biopharmaceutics and Drug Disposition, **11**, 311-350 (1990)
2) Analysis of Buprenorphine in Plasma and Urine by Gaschromatography., Martinez D. et. al., Journal of Chromatography, **528**,459-463 (1990)
3) Analysis of Buprenorphine and Its N-Desalkylated Metabolite in Plasma and Urine by Selected-Ion Monitoring., Journal of Chromatography, **338**, 89-98 (1985)
4) Quantitative Determination of Buprenorphine and Its Active Metabolite, Norbuprenorphine, in Human Plasma by Gaschromatography-Chemical Ionization Mass Spectrometry., Ohtani M. et. al., Journal of Chromatography, **487**, 469-475 (1989).

Acknowledgemt
This research was granted by The Japan Amateur Sports Association and by The Japan Cycling Promotion Association. Thanks are due to Dr. Shiraishi for providing the reference standard of BUP, NBP and CMBP.

In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994

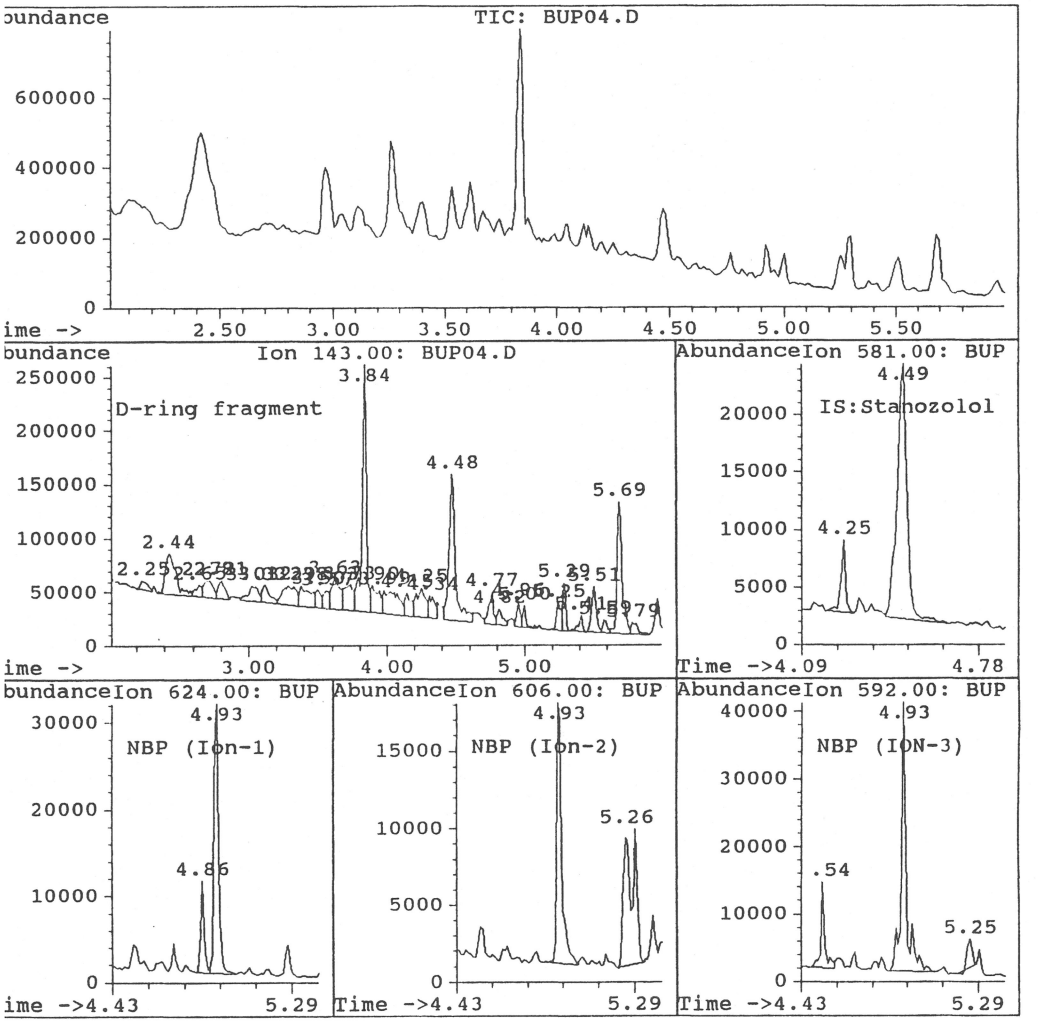


Figure-1. Identification of Urinary Buprenorphine as Norbuprenorphine-O-TMS-N-HFB in the Free Steroid Fraction.
(The result obtained from the urine collected at 9 hrs after oral administration)

In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994

Monitoring Ions and Relative Retention Time of Main Metabolites in Free Fraction

Steroids	*Metabilites*	*Derivative*	*Relative RT*	*Monitoring Ions (m/z)*		
Buprenorphine	Norbuprenorphine	TMS/HFBA	1.100	592	606	624
Cl-Dehydromethyl-testosterone	6b-OH-Cl-dehydromethyltestosterone	bis-TMS	1.007	143	315	317
Fluoxymesterone	17a-CH3-androst-4-en-9a-F-3a,6b,11b,17b-tetrol	tetra-TMS	0.908	143	552	642
Metandienone	6b-OH-metandienone	bis-TMS	0.921	143	281	460
	17-epi-6b-OH-metandienone	bis-TMS	0.857	143	281	460
Oxandrolone	parent compound	TMS	0.896	143	308	321
	17-epi-oxandrolone	TMS	0.835	143	308	321
Stanozolol	3'-OH-stanozolol	bis-TMS-N-HFB	1.087	143	669	684
	3'-OH-17epi-stanozolol	bis-TMS-N-HFB	1.008	143	669	684
Internal STD	Stanozolol	TMS-N-HFB	1.000	581	586	

Ions deleted by line were used for the monitoring of an interference.

Abbreviations: RRT = relative retention time, a = alfa, b = beta, CH3 = methyl, TMS = trimethylsilyl, OH = hydroxy

In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994

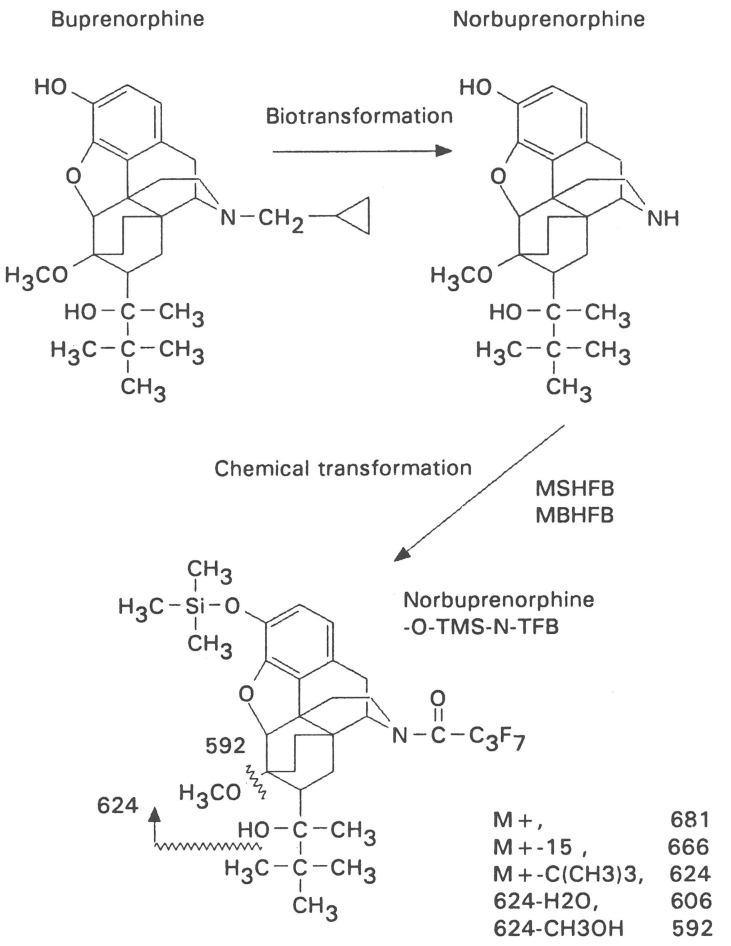
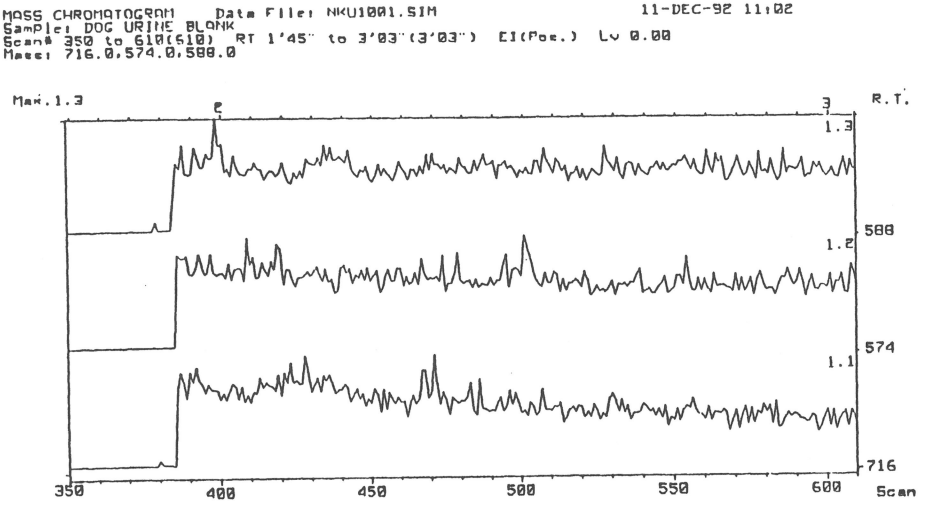
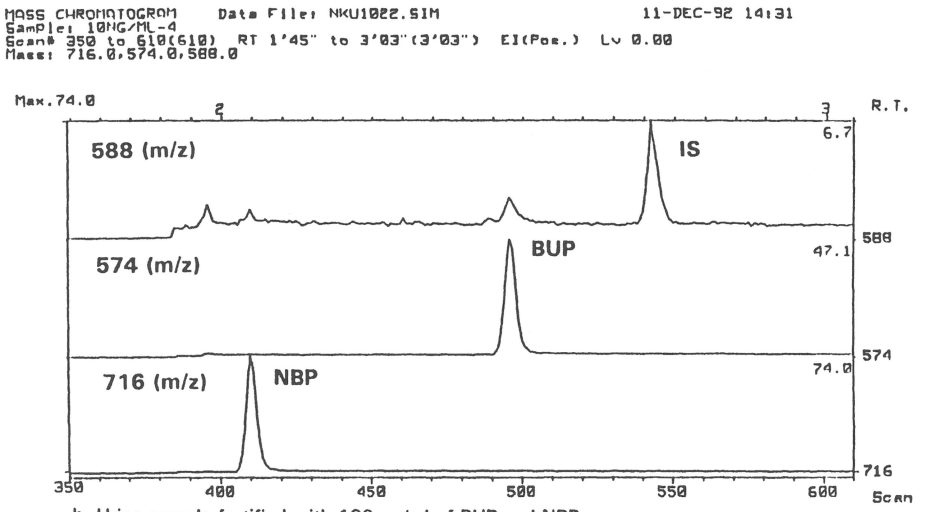


Figure-2 Identification of Buprenorphine as the Active Metabolite Norbuprenorphine.

In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994



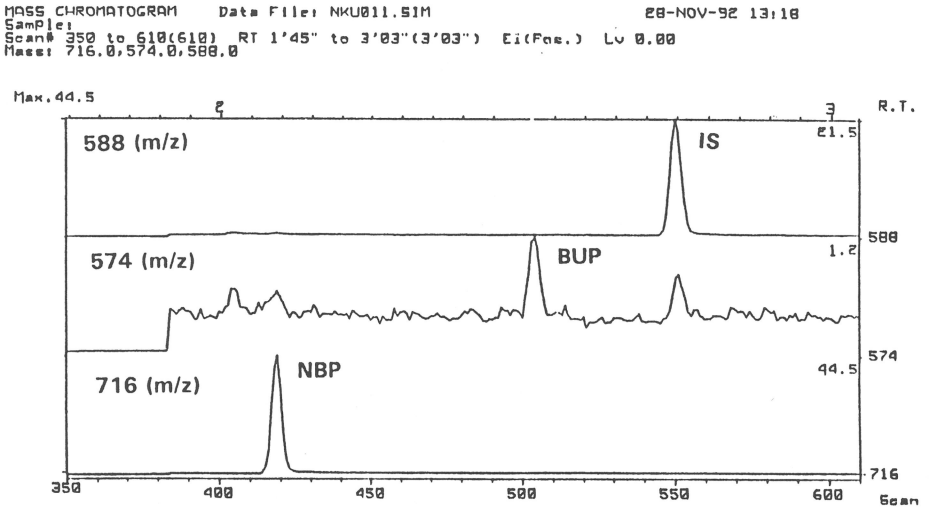
a. Water Blank



b. Urine sample fortified with 100 pg/ml of BUP and NBP

Figure-3. Quantitative analysis of Buprenorphine (BUP) and Norbuprenorphine (NBP) by GC/MS
(a. Water Blank, b. Urine sample fortified with 100 pg/ml of BUP and NBP)

In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994



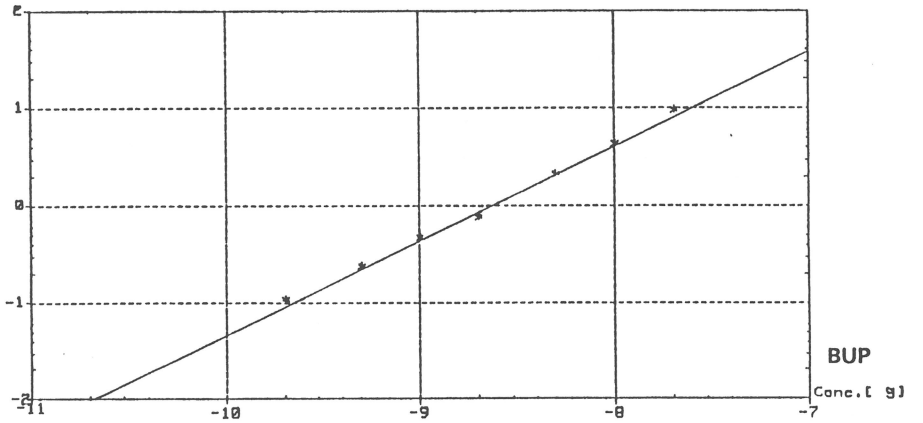


Figure-4. Quantitative analysis of Urinary Buprenorphine (BUP) and Norbuprenorphine (NBP) by GC/MS
(The result obtainned from a urine sample collected at 9hrs after oral administration)

In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994