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"Simultaneous Determination and Quantitation of Cocaine Metabolites in Urine by GC/MS. Could Possibly Some of Them Be Used as New Reliable Markers of Cocaine Ingestion in Addition to Benzoylecgonine?"

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Introduction

According to 1998 Chilean survey, more than 400 thousand Chileans age 12 to 64 have tried cocaine at least once in their lives. About 150 thousand had used cocaine the previous survey`s year. Cocaine is specially used in the cheap semi-refined form "free base"* known to be highly addictive and named "angustia" by the users due to the distinctive withdrawal symptoms (1). Concern about the problem has led to develop and implement drug abuse programs and drug testing policies to assess the nature and extent of drug abuse.

Benzoylecgonine (BZE) is the mayor metabolite of cocaine in urine and its presence is used to confirm the recent use of cocaine. However BZE is a transformation product that could be generated by carboxylesterases enzymatic pathway but also by in vitro chemical hydrolysis. This fact could perfectly be used to discuss the presence of BZE in positive cocaine urine samples in drug of abuse and/or doping control programs. For this reason a sensitive and rapid GC/MS macro SIM method was developed and validated for the detection and quantitation of BZE in the presence of one or several metabolites, to discard allegations of contamination of the urine with cocaine and corroborate that BZE evidence is the result of cocaine ingestion and not external contamination.

(*) Free base ("pasta base") is consumed straight or in mixture with marijuana ("marciano") and with tobacco ("tabacazo")

Materials and methods

Chemicals and reagents

Solvents and reagents were analytical or HPLC grade. Standars reference: Cocaine (COC), Norcocaine (NorCOC), Benzoylecgonine (BZE), Benzoylecgonine -d3 (BZE-d3), ecgonine methyl ester (EME), norbenzoylecgonine (NorBZE), m-hydroxybenzoylecgonine (m-OHBZE) and p-hydrxybenzoylecgonine (p-OHBZE), cocaethylene (CE), Norcocaethylene (NorCE), ecgonine (EC) and anhydroecgonine methyl ester (AEME) were purchased from Radian (Austin Texas, USA); m-hydroxyCocaine (m-OHCOC) and p-hydrxyCocaine (p-OHCOC) from Elsohly Laboratories (Oxford, Mississippi, USA).

The derivatizing reagents, MSTFA was purchased from Aldrich Chem. Co. and TMSCl from Merck (Darmstadt, Germany).

Negative urines were certified by GC/MS after Elisa negative test. Elisa inmunoassay (Neogen Corporation. Lexington, K.Y.USA) for Cocaine/benzoylecgonine supplied by H.L.S Veterinary Biological Products Inc.(Hillsdale. USA) and TDx Analyser FPIA system from Abbott Laboratories Diagnostics Division (IL. USA) supplied by Abbott Laboratories of Chile Ltda.

Forensic specimens

Twenty eight urine samples that had been collected and screened positive for BZE using Elisa immunoassay (Neogen, Co) Cocaine/benzoylecgonine for doping samples and TDx Analyzer System fluorescent polarization inmunoassay (FPIA) for drug of abuse samples, were used for this study.

Methods

Urines certified to be negative for cocaine and metabolites by GC/MS were pooled and used in the preparation of a duplicated calibration curve (25, 50, 100, 150, 300, 600 and 1000 ng/ mL of BZE) and quality controls (50, 150 and 500 ng/mL of BZE) used in each run.

To 5 mL aliquots of each specimen or quality controls were added 75 μL of BZE-d3 working internal standard which resulted in an analytical concentration of 150 ng/mL. Aliquots of 5 mL of both samples were applied over solid phase column copolymeric resin extraction cartridge (Clean Screen ZSDAU020 United Chemical Technologies) previously conditioned with 3 mL methanol, 3 mL of dionized water and 1 mL of 100mM phosphate buffer (pH=6). Columns were washed with 2 mL of water, 2 ml of HCL 100 mM and 3 ml of methanol and then eluted with 3 ml of dichlorometane: isopropyl-alcohol: ammonium

hydroxyde (78:20:2) (2). The resulting extracts were evaporated to dryness and the residues were derivatized with 100 µL of MSTFA/TMSCl 1 % by heating 30 min at 75 °C. The derivatized extracts were cooled at room temperature and 2µL injected into Hewlett Packard 6890/5973 GC/MSD operated in the SIM mode (table N° 6) using a Macro. The injector in splitless mode was operated at 250°C. The separation was carried out using a polydimethylsiloxane fused silica capillary column (HP1, 25 m., 0,2 mm i.d. and film thickness 0,11 µm). The temperature program was: initial temperature at 80 °C, rate 15°C/min to 310 °C and mantained for 1,5 min. Helium was used as a carrier gas at 0,8 ml/min.

Results and Discussion

- The urine samples analyzed covered a wide range of BZE concentrations from 4 ng to 92500 ng/mL.
- The analysis of BZE occurred independently of the analysis of NorBZE, m-OHBZE and p-OHBZE, because of the high concentrations of BZE relative to other metabolites.
- The LOD and LOQ of the method for BZE were determined by analyzing three replicates of a negative urine spiked at decreasing concentrations from 25 to 0.5 ng/mL. The resulting LOD and LOQ for BZE were 5 ng/mL.
- Extraction recoveries were performed for duplicated quality controls 50, 150 and 300 ng/mL. The results were 102%, 101% and 102% respectively.
- Precision and accuracy of the procedure were performed inter-day and intra-day study for duplicated quality controls 50, 150 and 500 ng/mL. (Table N° 7).
- Urines with BZE concentration higher than 1000 ng/mL have to be diluted to adjust to the calibration curve ranging from 25 to 1000 ng/mL.
- The implication that BZE could be formed by in vitro chemical hydrolysis aside of being generated by enzymatic pathway, has resulted in allegations of urine contamination during the collection step or the matrix analysis (3).
- Other metabolites wich were detected, in fewer samples included, m-OHCOC, p-OHCOC, NorCOC, m-OHCOC, p-OHCOC, CE, NorCE, ECG, and AEME a pyrolisis product and a marker for smoked cocaine, the general trend was that a greater number of metabolites were detected in higher BZE urine concentrations.

Table N°1, Drug of abuse urines with high concentrations of BZE (from 2000 to 92500 ng/mL): There is a complete profile of metabolites with no identification problem. The prevalence of NorBZE, m-OHBZE and p-OHBZE in urines with BZE concentration higher than 2000 ng/mL was 100%. Considering the prevalence of each of the three metabolites, 100% of the samples could be classified as positives.

Table N°2, Drug of abuse urines over an arbitrary cutoff of 100 ng/mL (between 130 and 595 ng/mL): The profile of metabolites ranged from 83% for NorBZE and p-OHBZE to 100% for m-OHBZE.

Table N°3, Drug of abuse urines with low concentrations of BZE, below cut-off of 100ng/mL (from 12 to 77 ng/mL): The profile of metabolites ranged from 0% for NorBZE, 50% for p-OHBZE to 100% for m-OHBZE.

Table N°4, Doping samples 1, concentration of BZE from 580 to 1300 ng/mL: The profile of metabolites ranged from 66% for NorBZE, to 100% for p-OHBZE and m-OHBZE.

Table N°5, Doping samples 2, concentration of BZE from 4 to 84 ng/mL: The profile of metabolites ranged from 0% for NorBZE, 33% p-OHBZE to 67% for m-OHBZE.

Considering the prevalence of each of the three metabolites, two of the urine samples (BZE concentration of 4 and 12 ng/mL) could be challenged on the assumption that the subject's urine was contaminated with cocaine during the collection step or drug analysis procedure, because there was no detection of any of the metabolites

- One limitation of this study is that the exact cocaine use histories of the users are not known. As there's data suggesting that regular use of cocaine appears to alter the disposition and elimination of cocaine when compared to occasional use (4), could be important to study urinary excretion pattern of both groups.
- The more prevalent metabolite in urines with low concentrations of BZE is m-OHBZE ranging from 67% to 100%, compared to 33% to 50% for p-OHBZE and 0% for NorBZE.
- The comparison of our results show almost full coincidence with those of Klette et al.(3) and J. Oyler et al.(5).(table N° 8,9).

Conclusions

- To discard allegations, the simultaneous analysis of all the three metabolites NorBZE, m-OHBZE and p-OHBZE, could be the response because strongly increases the chances of establishing the ingestion of cocaine specially the presence of m-OHBZE. Findings of BZE with either of the three mentioned metabolites in our opinion are conclusive of cocaine consumption.

References

1.- CONACE: Tercer Estudio Nacional sobre el Consumo de Drogas en Chile (1998).

2.-Worldwide Monitoring Corporation Clean Screen Extraction Column. Application Manual (1997).

3.- Kevin L. Klette, Gregory K. Poch, and Robert Czarny: "Simultaneous GC/MS Analysis of meta-and para-Hidroxybenzoylecgonine and Norbenzoylecgonine: A Secondary Method to Corroborate Cocaine Ingestion Using Nonhydrolytic Metabolites". J. Anal. Toxicol. 24: 482-488 (2000).

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5.- Jonathan Oyler, William D. Darwin, Kezie L.Preston, Patricia Suess and Edward J.Cone: "Cocaine Disposition in Meconium from Newborns of Cocaine-Abusing Mothers and Urine of Adult Drug Users". J. Anal. Toxicol. 20: 453-462 (1996).

Table N°1. Drug of Abuse (High BZE Samples, between 2000 and 95000 ng/ml)

Sample Code	BZE	Nor BZE	m-OH BZE	p-OH BZE	COC	Nor COC	m-OH COC	p-OH COC	CE	NorCE	ECG	AEME	EME	EEE
2301	10150	D	D	D	D	D	D	D	D	D	D	D	D	D
967	80750	D	D	D	D	D	D	D	N.D	D	D	D	D	D
2262	92500	D	D	D	D	D	D	D	D	D	D	D	D	D
2265	55000	D	D	D	D	D	D	D	N.D	D	D	D	D	D
2274	40500	D	D	D	D	D	D	D	D	D	D	D	D	D
2367	7100	D	D	D	D	D	D	D	D	D	D	D	D	D
2140	3500	D	D	D	D	D	D	D	D	D	D	D	D	D
2368	2080	D	D	D	D	D	D	D	D	D	D	D	D	D
2372	2372	D	D	D	D	D	D	D	D	D	D	D	D	D

Table N°2. Drug of Abuse (Positive BZE Samples, over a cutoff 100 ng/ml, between 130 and 595 ng/ml)

Sample Code	BZE	Nor BZE	m-OH BZE	p-OH BZE	COC	Nor COC	m-OH COC	p-OH COC	CE	NorCE	ECG	AEME	EME	EEE
2366	595	D	D	D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	D	D
2369	387	D	D	D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
2314	370	D	D	D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	D	D
2381	227	D	D	D	D	D	N.D	N.D	N.D	N.D	N.D	N.D	D	D
2298	215	D	D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	D	N.D
2371	131	N.D	D	D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	D	N.D

Table N°3. Drug of Abuse (Low Concentration Samples, below cutoff of 100 ng/ml)

Sample Code	BZE	Nor BZE	m-OH BZE	p-OH BZE	COC	Nor COC	m-OH COC	p-OH COC	CE	NorCE	ECG	AEME	EME	EEE
2247	77	N.D	D	D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	D	N.D
2143	57	N.D	D	N.D	D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	D	N.D
2112	13	N.D	D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	D	D
2269	12	N.D	D	D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	D	D

Table N°4. Doping Samples 1

Sample Code	BZE	Nor BZE	m-OH BZE	p-OH BZE	COC	Nor COC	m-OH COC	p-OH COC	CE	NorCE	ECG	AEME	EME	EEE
1399	1300	D	D	D	D	N.D	D	N.D	N.D	N.D	N.D	D	D	D
771	1420	D	D	D	D	N.D	D	N.D	N.D	N.D	N.D	D	D	D
1170	580	N.D	D	D	D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	D	N.D

Table N°5. Doping Samples 2

Sample Code	BZE	Nor BZE	m-OH BZE	p-OH BZE	COC	Nor COC	m-OH COC	p-OH COC	CE	NorCE	ECG	AEME	EME	EEE
3582	84	N.D	D	D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	D	D
636	50	N.D	D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	D	D
1845	28	N.D	D	D	D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	D	D
3687	22	N.D	D	N.D	N.D	N.D	D	N.D	N.D	N.D	N.D	N.D	D	N.D
3601	12	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
664	4	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D

Detected : D
Not Detected : N.D

Abbreviations:

BZE, Benzoylecgonine; NorBZE, norbenzoylecgonine;m-OHBZE, m-hydroxybenzoylecgonine; p-OHBZE, p-hydroxybenzoylecgonine; COC, Cocaine; NorCOC, norcocaine; m-OHCOC, m-hydroxycocaine; p-OHCOC, p-hydroxycocaine; CE, cocaethylene; NorCE, norcocaethylene; ECG, ecgonine; AEME, anhydroecgonine methyl ester; EME, ecgonine methyl ester; EEE, ecgonine ethyl ester.

Table N°6. Derivatived formed, monitored selective ion and metabolites retention time.

Metabolite of cocaine	Ion 1	Ion 2	Ion 3	Retention time
Benzoylecgonine (TMS)	82	240	361	10,72
Norbenzoylecgonine (diTMS)	140	298	404	11,32
m-hydroxybenzoylecgonine (diTMS)	240	434	449	12,40
p-hydroxybenzoylecgonine (diTMS)	193	434	449	12,68
Cocaine	82	182	303	10,30
Norcocaine (TMS)	140	346		10,85
m-hydroxycocaine (TMS)	182	360	391	12,04
p-hydroxycocaine (TMS)	182	360	391	12,39
cocaethylene	196	317		10,61
Norcocaethylene (TMS)	140	360		11,11
Ecgonine (diTMS)	96	314	329	6,74
anhydroecgonine methyl ester	152	181	122	4,86
ecgonine methyl ester (TMS)	82	96	271	6,18
ecgonine ethyl ester (TMS)	96	196	285	6,59

Table N°7. Inter-day and intra-day precision and accuracy for benzoylecgonine.

Inter-day run	Quality controls of BZE theorical concentration ng/mL		
	50	150	500
Mean (n=6)	50	148	490
SD n-1	3.293	4.605	21.80
c.v	6.599 %	3.110 %	4.257 %
DMT	-----	-1.33	- 2 %
Intra-day run	Quality controls of BZE theorical concentration ng/mL		
	50	150	500
Mean (n=10)	49	151	509
SD n-1	2.811	1.329	7.973
c.v	5.678%	0.879 %	1.565 %
DMT	6 %	0.667 %	1.8 %

SD: standard deviation: C.V, coefficient of variation: DMT, deviation of mean value from nominal

Table N°8.- Ranges of benzoylecgonine concentration (ng/mL) in urine.

Reference	Number of samples	range	Mean
Klette et.al	89	34 - 100,118	2,968
J.Oyler et.al.	34	43110 – 1,178,900	272,435
Báez et. al.	28	4 – 92,000	10,697

Table N°9.-Percent prevalence of the three BZE metabolites.

Reference	Nor BZE	mOHBZE	pOHBZE
Klette et.al.	67 %	83 %	89 %
J.Oyler et. al.	100 %	97 %	100 %
Báez et. al.	45 %	92 %	75 %