The simultaneous determination of cocaine and two breakdown products in oral fluid by LC-MS-MS

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ABSTRACT

This paper describes the use of liquid chromatography with a tandem mass spectrometric detection (LC-MS-MS) for the identification and quantitation of cocaine and cocaine metabolites or breakdown products in oral fluid. Samples were collected on arrest using the Cozart® RapiScan Collection System for oral fluid. The analytes of interest were extracted by solid phase extraction (SPE) using a mixed mode SPE cartridge. Analysis was carried out using a Varian LC-MS-MS. Each analyte was determined by multireaction monitoring (MRM) of two transitions per ions (cocaine m/z 304 to 182 and 105; benzoylecgonine m/z 290 to 168 and 105; cocaethylene m/z 318 to 196 and 150). Deuterated internal standards were used for the quantitation of each analyte. Calibration standards at 0, 15, 30, 60, 120 and 180 ng/mL were used and each sample, standard and control was spiked with deuterated internal standard at 120 ng/mL. The method is linear over the range LOQ to 180 ng/mL. The detection limit (LOD) is 1 ng/mL and the quantitation limit (LOQ) is 2 ng/mL for each analyte. The assay drift was less than 3%. The retention time for the peaks of interest varied from 0.16% to 0.33% and the transition ion ratios varied from 2.2% to 5.7% within run. The intra-assay precision was approximately 3% and the inter-assay precision, 4%. Following analysis of 437 oral fluid samples using this method, with a cut-off of 8 ng/ml, 53.5% were negative, 28.8% were positive for benzoylecgonine alone, 17.2% were positive for benzoylecgonine and cocaine; 0.5% were positive for all three analytes.
In conclusion, this method is suitably sensitive and robust for the routine analysis of cocaine and metabolites in oral fluid. There is scope for future development of this method to include the pyrolysis products of cocaine present following the use of crack cocaine and to decrease the sample volume from the 200 µL currently used for analysis.
Keywords: oral fluid, cocaine, benzoylecgonine, cocaethylene, LC-MS-MS

INTRODUCTION

The analysis of drugs of abuse in oral fluid poses a challenge due to limited sample volume available for analysis and the requirement to detect drugs at lower concentrations. The advent of tandem mass spectrometric detection techniques has enabled analysts to meet both these challenges [1-5]. Liquid chromatography tandem mass spectrometry (LC-MS-MS) has even been suggested as screening test for drugs of abuse in oral fluid [6]. The aim of the work described here was to develop and validate a sensitive and robust LC-MS-MS method suitable for the routine analysis of cocaine, benzoylecgonine and cocaethylene in oral fluid.

Cocaine appears in oral fluid immediately after drug administration by intravenous injection [7]. Benzoylecgonine and ecgonine methyl ester appear in oral fluid within 15 minutes of intravenous cocaine administration and are found at concentrations similar to those found in blood. In unstimulated saliva cocaine is ion-trapped in saliva and the saliva/plasma ratio may be five or greater [8]. n stimulated saliva, the saliva/plasma ratio ranges from 0.5 to 3.0 [9-13]. Jenkins et al [12] reported saliva/plasma ratios for benzoylecgonine ranging from 0.02 to 0.66. Cocaethylene is found in oral fluid when ethanol is ingested concurrent with cocaine.   In a rat model Barbieri et al [14] reported a saliva/plasma ratio of 1.3 for cocaethylene after cocaethylene administration.

Published data from single dose clinical studies show that cocaine and cocaine breakdown products can be detected in oral fluid up to 8 hours after an intravenous injection and 12 hours after smoking cocaine [12, 15]. Cone et al [9] reported an average half-life of cocaine of 34.7 min in oral fluid and 34.9 min in plasma after intravenous cocaine administration. Oral fluid terminal half-lives were 7.9 hours for cocaine, 9.2 hours for benzoylecgonine and 10 hours for ecgonine methyl ester. Due to the administration of single doses, these studies may well underestimate the detection times experienced from samples collected in more realistic situations. Jufer et al [16] reported prolonged terminal elimination half-lifes and extended detection times (fourfold for cocaine and sevenfold for benzoylecgonine) for cocaine and metabolites in oral fluid after repeated administration of cocaine in a controlled study, due to accumulation of lipophilic drug. Higher concentration levels of cocaine and breakdown products, and prolonged detection times of up to 5 days have been reported in data from a study performed on behalf of the UK government [17] and our own data from regular cocaine users [Wilson unpublished].

For long term determination of exposure to cocaine using oral fluid, a confirmation cutoff of 8 ng/ml cocaine or benzoylecgonine has been recommended [18, 19]. A goal in the development of this LC-MS-MS method was to achieve a limit of quantitation below 8 ng/ml cocaine in oral fluid and to validate this method sufficiently to meet ISO 17025 requirements for accredited laboratory use. The validation of the method involved determination of linearity, detection and quantitation limits, robustness, assay drift, and the precision of the method. The method was used for the analysis of 437 samples from donors sampled on arrest.

METHODS

Sample Collection

Oral fluid samples were collected from 437 donors who were sampled on arrest.   All samples were collected using the Cozart® RapiScan Collection System for oral fluid. One milliliter of oral fluid was collected from each subject on each occasion using the Cozart® Rapiscan Oral Fluid Collector. The collector has an indicator in the plastic handle that turns blue when one milliliter of fluid is collected. The oral fluid-soaked collector pad was placed in the Cozart® RapiScan Collector test tube with 2 ml of run buffer giving a final 1:3 dilution of the oral fluid. All concentrations cited in this paper are corrected for this dilution and expressed as ng/ml neat oral fluid. The tubes of oral fluid-buffer mixture were capped and sent by post to the Analytical Services Division within Cozart Bioscience where the diluted oral fluid was tested using

Cozart® Microplate EIAs for opiates, cocaine and specific morphine. After confirmation by GC-MS and/or LC-MS-MS any remaining fluid was stored frozen at -20°C.

Sample Preparation

For LC-MS-MS analysis, calibration standards were prepared by adding 0.2 mL blank oral fluid buffer mixture to a vial. Then appropriate amounts of standard mixtures of cocaine, benzoylecgonine and cocaethylene were added (100 ng/mL and 1000 ng/mL) to make the following neat oral fluid equivalent spiked concentrations: 0, 15, 30, 60, 120 and 180 ng/ml. 40 microlitres deuterated internal standard mixture was added to standards, controls and samples (0.2 mL mixed oral fluid and buffer) using 1000 ng/mL stock solution (cocaine-d3, benzoylecgonine-d3 and cocaethylene-d3) to make a final concentration equivalent to 120 ng/mL in neat oral fluid.

Solid Phase Extraction

The samples were extracted by solid-phase extraction (SPE) using mixed mode SPE cartridges (BondElut Certify 50mg, 3mL) and a VacMaster SPE vacuum manifold (IST Ltd, UK). The columns were conditioned with 1 mL methanol followed by 1 mL phosphate buffer (pH 7.4, 0.1 M). To 200 µL of sample, 1 mL pH 6 phosphate buffer was added and the samples (including deuterated-internal standards) were then loaded onto the columns and washed with 1 mL de-ionised water. The cartridge was then washed with 0.5 mL of 0.01 M HCl, dried and washed further with 2.0 mL of methanol. The cartridges were then dried on full vacuum for 10 min. Cocaine and its two breakdown products were eluted with 1 mL dichloromethane:methanol containing 2% v/v ammonia (88:10:2, v/v). The eluates were evaporated to dryness under nitrogen at 40°C and then reconstituted in 200µL 0.1% v/v acetic acid in 10% v/v methanol (mobile phase). 20 µL were injected onto the LC-MS-MS.

LC MS-MS Method Parameters

Liquid chromatographic analysis was carried out using a Varian Prostar 210 LC, 410 AS, using a Polaris C18-ether column (3µ x 50 mm x 2mm). The Mobile Phase A was 0.1%v/v acetic acid and Mobile Phase B was 0.1% v/v acetic acid in methanol. An elution gradient (10% mobile phase B increasing to 40% B over 8 minutes, then to 90% B after 10 minutes, returning to 10% after 10.5 minutes with a 6.5 minute equilibration time) at a flow rate of 0.25 mL/min was used for a run time of 17 min. The column temperature was maintained at 40°C.
The tandem mass spectrometer (MS-MS) was a Varian 1200L employing electrospray ionization. The drying gas temperature was 300°C and the pressure was 60 psi. The nebulizing gas pressure was 18 psi. Capillary voltage was 70V and collision gas pressure was 1.6 m Torr. Each analyte was determined by multireaction monitoring (MRM) of two transitions per ion. These were for cocaine m/z 304 to 182 and 105; for benzoylecgonine m/z 290 to 168 and 105; and for cocaethlyene m/z 318 to to 196 and 150 (Table 1 and Figure 1). For the internal standards the transitions were cocaine-d3 m/z 307 to 185; for benzoylecgonine-d3 m/z 293 to 171; and for cocaethlyene-d3 m/z 321 to 199.

The abundances found for the following ions relative to the internal standard abundances were used for quantitation: benzoylecgonine m/z 168, benzoylecgonine-d3 171; cocaine m/z 182, cocaine d-3 185; cocaethylene m/z 196, cocaethylene-d3 199.

Table 1: MRM Settings

Analyte	Ion Transitions	Collision Energy electron volts
Benzoylecgonine	**m/z 290 => 168**	**-14.5**
	m/z 290 => 105	**-24.5**
Benzoyleconine-d3	**m/z 293 => 171**	**-14.5**
Cocaine	**m/z 304 => 182**	**-15**
	m/z 304 => 105	**-26**
Cocaine-d3	**m/z 307 => 195**	**-15**
Cocaethylene	**m/z 318 => 196**	**-15**
	m/z 318 => 150	**-20.5**
Cocaethylene-d3	**m/z 321 => 199**	**-15**

Matrix Controls

Oral fluid samples previously analyzed were repeated on subsequent runs as controls and the results compared to the previous values. Each batch of twenty samples included on blank oral fluid sample and two positive controls spiked at a concentration of approximately 20 ng/mL.

Linearity, Limit of Detection, Limit of Quantitation and Robustness

The linearity was determined by analyzing four separate calibration curves over the range of 2 to 180 ng/mL for each analyte. The mean and standard deviation of the correlation coefficients for the calibration curves were determined.
The limit of detection was defined as three times the background as determined by the Varian software as the signal to noise ratio. The limit of quantitation was the lowest level at which the analytes could be identified and quantified correctly. Both the limit of detection and quantitation were determined from spiked oral fluid specimens which were repeatedly diluted with blank oral fluid-buffer mixture and analyzed. The criteria for acceptable identification was ion ratios of the two product ions within 25% of those of the extracted calibration standard and the criterion for acceptable quantitation was that the result must be within 2 standard deviations of the predetermined value for the dilution. The criteria for the run were the calibration curve must have a correlation coefficient of greater than 0.99 and the analytical quality controls must be within two standard deviations of the pre-determined mean for those controls.
The robustness of the method was determined by looking at the variation (percent coefficient of variation) of the analyte retention times from the beginning to the end of an analytical run and the variation of the ratios of the two product ions in the MS-MS. Assay drift was determined by injecting a spiked control at the beginning and end of the analytical run and determining the percentage error.

Fig.1: Chromatograms for the analyte and internal standard MRM transitions for a.) blank oral fluid spiked at 60 ng/ml for each analyte, b.) blank oral fluid spiked at 60 ng/ml for each internal standard and c.) oral fluid collected from a donor which contained 110 ng/ml cocaine, 1328 ng/ml benzoylecgonine and 264 ng/ml cocaethylene.
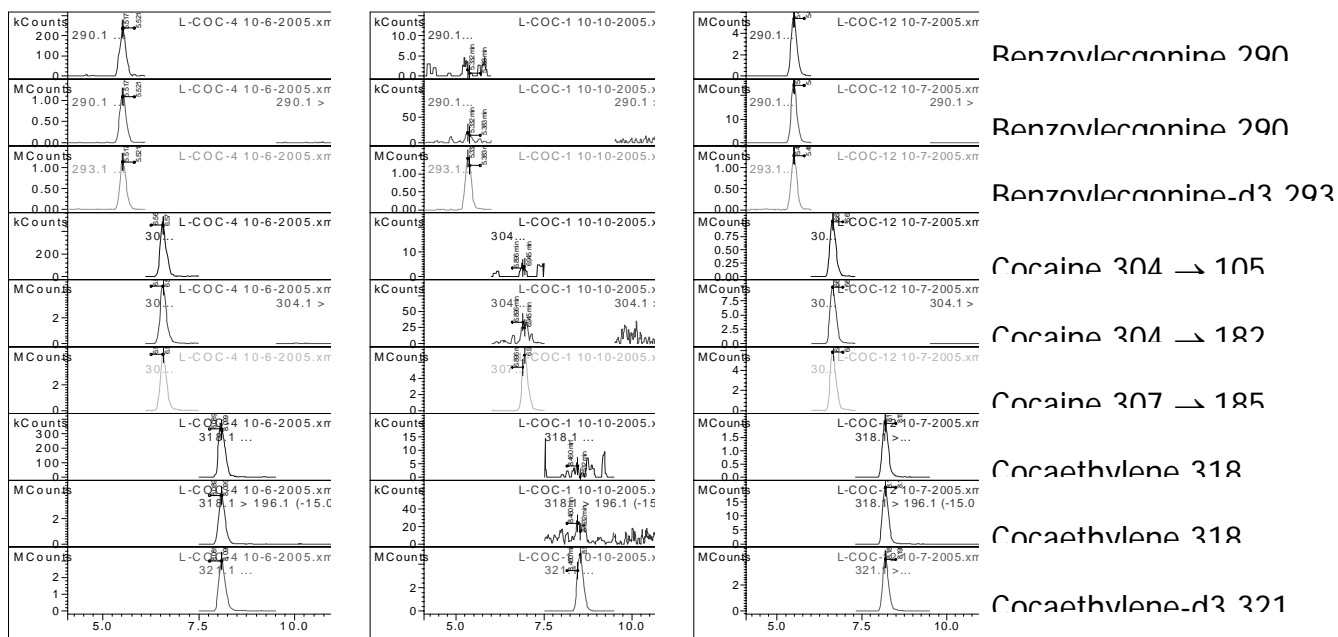
Precision

The precision of the method was determined by analysing four separate runs each containing ten aliquots of a spiked oral fluid sample at 30 ng/mL each cocaine, benzoylecgonine and cocaethylene. Precision over the forty analyses (inter-assay) and the ten analyses within one run (intra-assay) was obtained by calculating the mean, standard deviation (SD) and coefficient of variation (CV) for each.

RESULTS AND DISCUSSION

Precision

The within assay precision for the LC-MS-MS method for cocaine and breakdown products in oral fluid ranged between 2.0% to 3.5% CV (Table 2). The between assay precision, over the forty assays ranged between 3.7% to 4.4% CV (Table 2).

Table 2: Summary of Precision Results

	Inter-Assay Precision (n = 40)				Intra-Assay Precision (n = 10)		
Analyte	Mean Conc. (ng/ml)	Std Dev (ng/ml)	CV (%)		Mean Conc. (ng/ml)	Std Dev (ng/ml)	CV (%)
Benzoylecgonine	30.3	1.3	4.4		29.8	0.8	2.6
Cocaine	25.5	1.1	4.4		24.5	0.8	3.5
Cocaethylene	26.1	1.0	3.7		26.5	0.5	2.0

Fig. 2: The distribution of benzoylecgonine concentrations in 437 criminal justice oral fluid specimens.
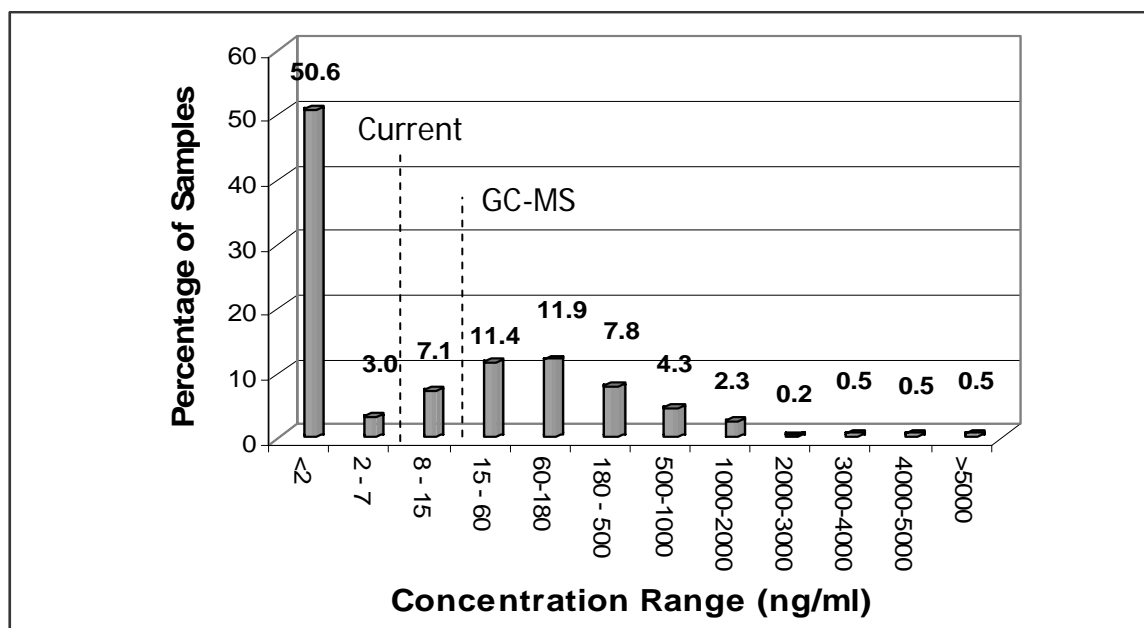
Limit of Detection and Limit of Quantitation

The limit of detection (LOD) of the method is 1 ng/mL cocaine, benzoylecgonine or cocaethylene and the limit of quantitation (LOQ) is 2 ng/mL for each analyte.   This is an improvement over the LOD and LOQ of the current GC/MS method of 15 ng/mL and meets the proposed SAMHSA confirmation test cutoff of 8 ng/mL cocaine or benzoylecgonine. Upon applying an 8 ng/mL cut-off routinely for this method an additional calibrator at 6 ng/ml will be included in the standard curve so that the cut-off level is bracketed by calibrators.

Linearity

The method is linear for each analyte over the range of LOD to 180 ng/mL. Over the range 2 ng/mL to 180 ng/mL the mean correlation coefficients (n = 4) were for cocaine 0.9990 ± 0.0006; for benzoylecgonine 0.9976 ± 0.0012 and for cocaethylene 0.9992 ± 0.007.

Robustness

The retention time of the peaks of interest had a within-run coefficient of variation of 0.24% for cocaine, 0.33% for benzoylecgonine, and 0.16% for cocaethylene. The within-run transition ion ratio coefficient of variation was 2.4% for cocaine, 5.7% for benzoylecgonine and 2.2% for cocaethylene. Therefore, the assay drift was less than 3%.

Donor Samples

Of 437 oral fluid samples collected on arrest of the donor and submitted for analysis, 234 were negative for cocaine and its breakdown products (53.5%), 126 contained benzoylecgonine only (28.8%), 75 contained both cocaine and benzoylecgonine (17.2%), 2 contained cocaine, benzoylecgonine and cocaethylene (0.5%). Ninety five samples were also positive for opiates in combination with cocaine and its breakdown products. Cocaine concentrations ranged from 2 to 1323 ng/mL, cocaethylene concentrations from 2 to 264 ng/mL and benzoylecgonine concentrations ranged from 6 to 12,613 ng/mL. The highest cocaine concentrations were associated with the highest benzoylecgonine concentrations. The distribution of benzoylecgonine concentrations is shown is Figure 2.

CONCLUSION

This method is suitably sensitive and robust for the routine analysis of cocaine and metabolites in oral fluid. Because of the low limit of quantitation the method can be used with reduced sample volume collection. There is scope for future development of this method to include the pyrolysis products of cocaine present following the use of crack cocaine.

References

1. K. A. Mortier, K.E. Maudens, W.E. Lambert, K.M. Clauwaert, J.F. Van Bocxlaer, D.L. Deforce, C.H. Van Peteghem and A.P. DeLeenheer, Simultaneous, quantitative determination of opiates, amphetamines, cocaine and benzoylecgonine in oral fluid by liquid chromatography quadrupole-time-of-flight mass spectrometry. J. Chromatogr B Analyt Technol Biomed Life Sci. 779 (2002) 321-330.
2. R. Dams, C.M. Murphy, R.E. Choo, W.E. Lambert, A.P. DeLeenheer and M.A. Huestis. LC-atmospheric pressure chemical ionization-MS/MS analysis of multiple illicit drugs, methadone and their metabolites in oral fluid following protein precipitation. Anal Chem. 75 (2003) 798-804.

3. K. Clauwaert, T. Decaestecker, K Mortier, W. Lambert, D. Deforce, C. Van Peteghem and J. Van Bocxlaer, The determination of cocaine, benzoylecgonine and cocaethylene in small-volume oral fluid samples by liquid chromatography-quadrupole-time-of-flight mass spectrometry. J Anal Toxicology 28 (2004) 655-659.

4. M. Wood, M. LaLoup, M. Ramirez-Fernandez, K.M. Jenkins, M.S. Young, J.G. Ramaekers, G. DeBoeck and N Samyn, Quantitative analysis of multiple illicit drugs in preserved oral fluid by solid-phase extraction and liquid chromatography-tandem mass spectrometry. Forensic Sci. Int. 150 (2005) 227-238.

5. H. H. Maurer, Advances in analytical toxicology: the current role of liquid chromatography-mass spectrometry in drug quantification in blood and oral fluid. Anal Bioanal. Chem 381 (2004) 110-118.

6. K.R. Allen, R. Azad, H.P. Field and D.K. Blake, Replacement of immunoassay by LC tandem mass spectrometry for the routine measurement of drugs of abuse in oral fluid. Ann Clin Biochem 42 (2005) 277-284.

7. L.K. Thompson, D. Yousefnejad, K. Kumor, et al, Confirmation of cocaine in human saliva after intravenous use. *J. Anal Toxicol.* 11 (1987) 36-38.

8. W. Schramm, P.A. Craig, R.H. Smith and G.E. Berger, Cocaine and benzoylecgonine in saliva, serum and urine. *Clin. Chem.* 39 (1993) 481-87.

9. E.J. Cone, K. Kumor, L.K. Thompson and M. Sherer, Correlation of saliva cocaine levels with plasma levels and with pharmacologic effects after intravenous cocaine administration in human subjects," *J. Anal. Toxicol.* 12 (1988) 200-206.

10. E.J. Cone and S.L. Menchen , Stability of cocaine in saliva. *Clin. Chem.* 34 (1988) 1508.

11. E.J. Cone and W. Weddington, Prolonged occurrence of cocaine in human saliva and urine after chronic use. *J. Anal. Toxicol.* 13 (1989) 65-68.

12. A.J. Jenkins, J.M. Oyler and E.J. Cone, Comparison of heroin and cocaine concentrations in saliva with concentrations in blood and plasma. *J. Anal. Toxicology*, 19 (1995) 359-74.

13. E.J. Cone, J. Oyler and W.D. Darwin, Cocaine disposition in saliva following intravenous, intranasal and smoked administration. *J. Anal. Toxicology* 21 (1997) 465-475.

14. E. J. Barberi, G.J. DiGregorio, A.P. Ferko and E.K. Ruch, Rat cocaethylene and benzoylecgonine concentrations in plasma and parotid saliva after the administration of cocaethylene," *J. Anal. Toxicol.* 18 (1994). 60-61.

15. E.T. Moolchan, E.J. Cone, A. Wstadik, et al. Cocaine and metabolite elimination patterns in chronic cocaine users during cessation: plasma and saliva analysis. *J. Anal. Toxicol.* 24 (2000) 458-466.

16. R.A. Jufer, S.L. Walsh, E.J. Cone and A. Sampson-Cone, Effect of repeated cocaine administration on detection times in oral fluid and urine. *J. Anal. Toxicol.* 30 (2006) 458-462.

17. D. Osselton, S. Robinson, A. Cox and S. Reddick, An evaluation of oral fluid drug testing for the detection of opiate and cocaine use in a population of drug users. Poster Presentation SOFT September 2001.

18. United Kingdom Laboratory Guidelines for Legally Defensible Workplace Drug Testing: 2. Oral Fluid Drug Testing. www.londontox.org.uk. 27 April 2005.

19. HHS Mandatory Guidelines: Notice of Proposed Revisions to Mandatory Guidelines for Federal Workplace Drug Testing Programs. 69 Federal Register April 13, 2004 pp.