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Determination of cocaine and its major metabolite benzoylecgonine in rabbit hair by GC/MS

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Abstract: It is important to determine people who use drugs to prevent the use of them. In recent years, hair is routinely used on toxicological analysis as well as biological samples such as blood and urine. The major practical advantages of hair testing are larger detection windows; non-invasive sample collection; easy to transport and store in addition hair analysis can provide a retrospective calendar of an individual's drug use history. In this study, Male New Zealand rabbits (n=10) weighing 2-2,5 kg were used. Two groups were constituted and each group has five rabbits. New Zealand rabbits were intraperitoneally administered with a single dose of cocaine. Following that hair samples were collected during the12th day. According to our data; cocaine is appealed the first five days after single dose of cocaine administration. Benzoylecgonine was not detected after three days. Frequency of benzoylecgonine existence was lower than cocaine. Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated for cocaine and benzoylecgonine. We did not any significant between groups.

Key Words: hair, cocaine, rabbit, forensic toxicology.

Blood and urine are the routine samples of choice for drug analysis. But in recent years, hair has been used as an alternative and fundamental biological sample in forensic and clinical toxicological analyses. The major practical advantage of hair testing is that it offers a larger detection window (from 3 days to years), depending on the length of the hair shaft; it provides information about the history of drug abuse of an individual its collection is not invasive; and it can easily be performed under conditions that prevent adulteration and substitution [1-2].

Hair grows in a cycle composed of the anagen, catagen and telogen stages. Anagen is the active growth phase where the cells in the bulb of a follicle are rapidly divided. During this phase the hair grows about 1cm every 28 days and stays in the anagen phase for 2-6 years. The catagen is a short transitional phase following the anagen phase. During this phase, which lasts for about 2-3 weeks, the cell division stops and the hair shaft becomes fully keratinized. The telogen phase is a resting phase in which the growth of the hair shaft stops completely. The hair is just anchored in the follicle by the root during this phase. The germ cells below the root will give rise to the next anagen-phase hair while the old hair will be forced out and lost. This phase lasts for about 10 weeks [3-4].

Drugs enter the hair by passive diffusion from blood capillaries into growing cells over a length of 1.2 to 1.5 mm between the level of matrix cells and the end of the keratinization zone of the hair follicle. Three important factors control the drug amount bound to protein inside the hair: 1) melanin affinity, 2) lipophilicity and 3) basicity of a drug. The rate of hair growth of a person depends on the anatomical site on which it grows, and the person's race, gender and state of health [2, 5-7].

The largest numbers of studies on hair analysis have dealt with drugs of abuse (especially cocaine,

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opiates and amphetamines). Cocaine (COC) is a potent drug, a stimulant that speeds up the activity of the brain and other parts of the central nervous system (CNS) and causes euphoria, hyperkinesia, the urge to talk, and an increasing readiness to take risks. Benzoylecgonine (BE) is the major metabolite of cocaine. It is of great toxicological interest due to its long half-life through which it remains detectable longer than cocaine itself [8]. Blood and urine analyses as biological samples are usually preferred in detecting the use of a single dose of COC. However, there are situations in which the use of these samples is not practical. Especially, victims of sexual assault can undergo severe psychological trauma. These victims may gain consciousness after hours or even days. So although the person appeals to the police in a few days, the blood and urine needed for toxicological analysis may not be enough to detect drugs. For this reason, and because of its advanced analytical sensitivity, hair analysis may be helpful in detecting a single dose of drug use [6].

Although the determination of abused drugs has been performed in human hair in many studies, this study was designed as an animal experiment because of the ethical problems involved in drug administration. Animal studies are helpful in understanding the mechanisms involved in the incorporation of drugs into hair.

The aim of the present study was to determine the detection of cocaine and its major metabolite BE in rabbit hair after a single-dose administration.

MATERIALS AND METHODS

Chemicals and reagents

All solvents and chemicals were of analytical grade (Merck, Germany). Reference standard solutions of COC, COC-d_3, BE and BE- d_3 were obtained from Cerilliant (USA). BSTFA was obtained from Supelco (USA).

Animal Study

Male New Zealand rabbits (n=10) weighing 2-2.5 kg were used. Two groups were constituted and each group had five rabbits. The animals were housed in metabolism cages with food and water ad libitum. Before drug administration blank hair of the rabbits was cut with an electric shaver, to be used as a control sample. Cocaine hydrochloride dissolved in normal saline was intraperitonally (i.p) administered in a single dose. The first group received 5mg/kg, and the second group 10 mg/kg. At least 50 mg of hair was collected during 12 days after drug administration. Hair samples were stored in a dry and dark condition at room temperature in aluminum foil until gas chromatography/mass spectrometry (GC/MS) analysis.

Cukurova University Medical Science Experimental Research Center obtained the research and ethical permissions on animal studies generally as well as specifically for this experimental study.

Sample Preparation

Each hair sample was washed to avoid drug detection arising from environmental contamination. The hair was sequentially washed with dichloromethane, deionized water and methanol. Each hair sample (20 mg) was weighed into a 10 ml glass tube. After this, 2 ml of methanol, 1 ml of 0.1 N hydrochloric acid and 20 µl of a standard mixture of the internal standard (10 µg/ml) were added and the mixture was incubated for 3h at 65°C [9]. After the incubation tube was centrifuged at 3500 rpm for 3 min, then the supernatant was transferred to another tube by a pastor pipette. Following this, 6 ml of 0.1M phosphate buffer was added. A solid phase cartridge (MCX) was used for sample clean-up. Firstly, an SPE cartridge was conditioned with 2 ml of methanol and 2 ml of deionized water. The sample was passed through the cartridge and washed with 2 ml of each of the following: deinonized water, 0.1 N hydrochloric acid, methanol: dichloromethane(1:1, *v/v*), and n-hexane, sequentially. The cartridge was dried under full vacuum for 1 min. Eluent was extracted with 2 ml of a mixture of dichloromethane/isopropanol (4:1, *v/v*) with 2% of ammonium hydroxide. After the extract were dried under nitrogen, 100µl of BSTFA: TMCS (99:1) was added and vortexed. Derivatization took place at 80°C for 25 min. A sample of 2 µl was injected into the GC/MS.

The standard calibration curve was obtained by blank hair with increasing amounts of COC and BE concentration levels ranging from 0.5 -100 ng/mg, all with 10 ng/mg COC-d_3 and BE-d3 added as internal standard, and the correlation coefficients were 0.999 and 0.997, respectively. The coefficient of variation was calculated at the lower calibration level (n=10) and it was 10 %.

Instrumentation

GC/MS analyses were performed with a Hewlett-Packard 6890N gas chromatography with a 5973 mass selective detector. The column was an HP-5 MS crosslinked 5% phenylmethyl-silicone fused–silica capillary column (30m x 0.25mm i.d.). The injection port temperature was 300°C, and the transfer line temperature was 280°C. The oven temperature was maintained at 90°C for 2 min, then at 20°C/min to 300°C and held for 4 min.

For quantitative analysis, the mass detector was operated in the selective ion monitoring (SIM) mode at *m/z* 82,182, 303 for cocaine; *m/z* 82, 240 and 361 for BE; and *m/z* 185 for COC-d3, *m/z* 243 for BE- d3.

RESULTS AND DISCUSSION

The number of publications describing methods

of testing for COC in hair has increased during recent years. Cocaine and its metabolites have been the target of hair tests varied by a different range of interests. Additionally, hair testing has been used in many fields, such as forensic medicine, traffic and occupational medicine, and clinical toxicology.

In our study the rabbits, weighing about 2-2.5 kgs, were divided into two groups and given two different concentrations (5 and 10 mg/kg) of cocaine. The reason for this was to find out whether a single dose could be detected in low quantities or not. Table 1 shows the quantity of COC detected in rabbit hair during the 12 days. While COC was detected in the first five days in both groups, no cocaine was found on subsequent days. Since cocaine metabolizes very quickly, in most biological samples, its major metabolite, benzoylecgonine is dominant. Yet the first metabolite found in hair is cocaine. The frequency of the existence of benzoylecgonine in hair is less. In the rabbits which were given 5 mg/kg cocaine, the cocaine concentration in hair on the 1st day was 1.73± 0.33.

Table 1. Cocaine concentration detected in rabbit hair.

	Group 1 (5mg/kg) n=5				Group 2 (10mg/kg) n=5				
Day	**X ± S.D.[a] (ng/mg)**	**Med**	**Min**	**Max**	**X ± S.D.[a] (ng/mg)**	**Med**	**Min**	**Max**	**p**
1	1.73± 0.33	1.68	1.42	2.22	1.18± 0.67	1.43	N.D.	1.69	0.175
2	1.64± 0.30	1.74	1.10	1.83	1.23± 0.71	1.46	N.D.	1.79	0.209
3	1.00± 0.58	1.22	N.D.	1.52	0.92± 0.52	1.06	N.D.	1.31	0.675
4	0.56± 0.76	N.D.	N.D.	1.41	0.68± 0.63	1.07	N.D.	1.18	0.911
5	1.06± 0.71	1.40	N.D.	1.44	0.55± 0.75	N.D.	N.D.	1.49	0.371

a: mean± standard deviation
N.D.: not detected

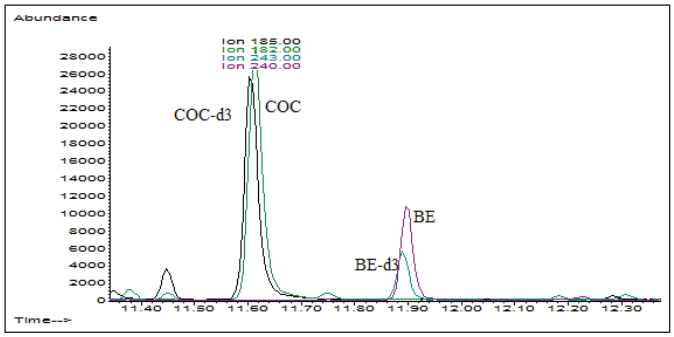

Figure 1. GC/MS chromatograms of 25ng/mg standard and internal standard.

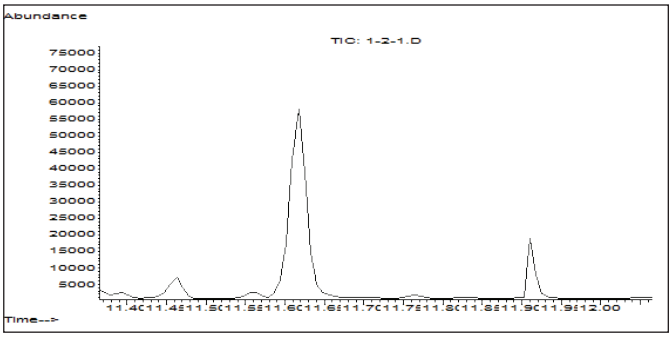

Figure 2. COC and BE peaks detected in rabbit hair.

Table 2. Frequency of existence of % BE.

Day	Cocaine Dose	
	5 mg/kg	**10mg/kg**
1	40	20
2	20	N.D.
3	40	40
4	N.D.	N.D.
5	N.D.	N.D.

However at the end of the 5th day it was 1.06± 0.71. In the rabbits which were given 10 mg/kg cocaine, the cocaine concentrations were 1.18± 0.67 on the 1st day and 0.55± 0.75 on the 5th day.

Figure 1 shows that both cocaine and benzoylecgonine were successfully identified in blank rabbit hair. Figure 2 shows cocaine and benzoylecgonine peaks found in rabbit hair after cocaine administration.

In this study, the penetration of the two different doses into the hair was almost the same. In other words, there were no differences observed between the groups ($p > 0.05$). These data also demonstrated that COC, and possibly BE, diffuse into hair forming cells of non-pigmented hair but rapidly diffuse out of the cells as the plasma analyte concentration falls. Jurado *et al.* have detected the highest dose of cocaine and benzoylecgonine in rabbits back hair on the 1st day by applying a single dose (5mg/kg) of cocaine to female Fauve Bourgogne rabbits. On other hand they have not found COC and its metabolite BE on the 10th day [10].

As BE was not observed in all rabbits given cocaine, a frequency table has been formed. Table 2 shows the frequency of existence of BE.

In the literature, when defining a dose-response relationship, the doses to be compared were administered over a 5-day period. Additionally, in controlled studies many drugs have been detected as preferably penetrating into pigmented hair.

Nakahara and Kikura examined the incorporation of COC, BE and ecgonine methyl ester (EME) into the hair of Dark Agouti rats. They concluded that the incorporation of COC from plasma into hair was

much greater than either BE and EME [11]. Hubbard *et al*. intraperitonally administrated three different concentrations of cocaine (5, 10, and 20 mg/kg) in Long Evans rats.

While cocaine concentration was detected in pigmented hair at the end of the 3rd day in rats administrated with 10 mg/kg cocaine, Benzoylecgonine was not detected on the 1st day. The COC and BE were detected in non-pigmented hair at the earliest time point. In non-pigmented hair, BE was only detectable from 1 to 4 h after dose administration in low concentration (≤ 0.18 ng/mg). The concentration of COC in nonpigmented hair was also low (≤ 0.23 ng/mg) and returned to zero by 2 days after dose administration [12].

The validity of blood and urine analyses relies on the elimination half-life of a specific substance. As such, if the drug to be detected is cocaine, whose elimination half-life is less than an hour, then one has to identify it before 24 hours because it is not possible to detect cocaine in blood or urine samples after a day or so. If the substance to be detected is cannabinoid or other narcotic drugs, however, they can be identified in blood or urine samples at a maximum of 3-4 weeks after administration [13,14].

It is difficult to interpret hair analysis results because many factors, such as lypofility, melanin affinity and pKa of drugs, affect the incorporation of drugs into hair. The Society of Hair Testing (SoHT) has proposed a number of guidelines for the analysis and interpretation of results of cocaine and metabolites in hair samples, stating that cocaine should be present at a concentration above the cut-off value of 0.5ng/mg and that at least one metabolite should be present at a concentration of 0.05ng/mg or higher [15,16]. In this study, the limit of quantification (LOQ) and limit of detection (LOD) for cocaine and benzoylecgonine were calculated for 0.1 and 0.3 ng/mg as 0.03ng/mg and 0.08 ng/mg, respectively.

In conclusion, this study demonstrated that cocaine can be detected throughout the initial 5 days after drug exposure. This detection duration is shorter in other biological samples such as blood and urine. In future experiments, we will conduct research on the pharmacokinetics of different cocaine concentrations in pigmented hair.

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