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Original article

Methyl paraben as a sex pheromone in canine urine – is the question still open?

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Abstract

The literature concerning the issue of canine sex pheromones includes reports presenting completely conflicting opinions about the chemical composition of the canine urine in the context of semiochemical communication. At present, the predominant report cited by many different authors is the article published in Science in 1979 by Goodwin at al., presenting methyl p-hydroxybenzoate (methyl paraben) as the main canine sex pheromone. While it has been proved that pure methyl paraben lacks semiochemical activity as do commercially available products containing this substance (Eau D'Estrus, Synbiotics, USA), in view of the conflicting published reports the aim of this study was to revaluate using modern techniques the presence of methyl p-hydroxybenzoate in canine urine during different phases of the ovarian cycle. Ten female dogs of different breeds were used. Urine samples from bitches collected during various stages of the ovarian cycle were examined with using the SPME and GC/MS methods. Methyl paraben was not detected in any of the samples. In conclusion, because of the lack of methyl-p-hydroxybenzoate in the samples examined, the present study confirmed negative opinions on the possibility of this substance playing a crucial role in semiochemical communication during reproduction in dogs (*Canis familiaris*).

Key Words: sex pheromones, bitch, GC/MS, methyl p-hydroxybenzoate, methyl paraben

Introduction

In the 1970's and 80's, there was a large interest in the topic of semiochemical communication in animals (Meredith et al. 1980, Novotny et al. 1984, Novotny et al. 1985, Raymer et al. 1986, Singer et al. 1986). While in the beginning rodents were the main group of animals tested for pheromonal communication, soon other species were investigated including wild and domestic animals (Brownlee et al. 1969, Izard and Vandenbergh 1982, Stevens et al. 1982, Raymer et al. 1986, Nishimura 1991, Jezierski 1992, Grzegorzewski 2006, Dzięcioł et al. 2012a, Dzięcioł et al. 2013). The rapid development of modern methods dedicated to detection and identification of chemical compounds, including gas chromatography-mass spectrometry (GC/MS), fostered studies into the composition of natural bioactive secretions.

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In 1979 Goodwin, Gooding and Regnier published in Science the results of their study describing sex pheromones in dogs (Goodwin et al. 1979). According to these authors, methyl p-hydroxybenzoate (methyl paraben, MP) is the main substance present in the urine of bitches in heat, and application of MP into the vulvar region of animals that were out of heat (anoestrus) stimulated males to exhibit mating behavior. This observation gave rise to a hypothesis that the main sex pheromone of bitches is just methyl paraben (Goodwin et al. 1979).

An attempt to verify this hypothesis was made by Kruse and Howard in 1983. In their paper published in the Journal of Chemical Ecology, they stated that even though they confirmed the presence of methyl paraben in samples of female dog urine, results of behavioral analysis showed that methyl p-hydroxybenzoate cannot be considered as a key sexual attractant for male dogs (Kruse and Howard 1983). It is also worth noting that in the article published by Schultz et al. (1985) the authors did not even include methyl paraben in the list of substances identified in samples of urine collected from bitches in oestrus.

While all the reports mentioned above on the issue of dog sex pheromones collectively presented contradictory conclusions, and therefore created great confusion in the area of canine sex pheromones, the aim of our work was to attempt once again to investigate the composition of canine urine and ascertain if methyl p-xydroxybenzoate can be considered as a dog sex pheromone. Because since the 1980's the accuracy of laboratory procedures like GC/MS has significantly improved, we decided to focus on confirming the presence of candidate substances in canine urine.

Materials and Methods

Location, animals and sample collection

The experiment was conducted in the Clinic of the Department of Reproduction and in the Laboratory of the Department of Chemistry belonging to the Wroclaw University of Environmental and Life Sciences, Poland. The study was approved by the Local Ethical Committee.

Samples were collected from bitches belonging to the Experimental Breeding Kennel located at the Department of Reproduction and from patients of the local Clinic of Reproduction. All animals included in the experiment were previously clinically examined and no diseases were found in any animals. Moreover, to eliminate the possibility of disease compromising the results, all urine samples were also tested for kidney, bladder and lower urinary tract diseases. Only samples from healthy females were used for further examination.

Samples of urine were collected from 10 bitches of different breeds (four beagles, four German shepherds, two golden retrievers) with an average age of 4-6 years. The phase of the oestrous cycle was determined according to the protocol described below. From each female, at least three samples of urine were collected during proestrus and three during oestrus. During these phases, the attractiveness of the females to the males was confirmed by performing tests with experienced male dogs belonging to the Experimental Kennel. In addition, urine samples were also collected from all of the females twice during anoestrus and metoestrus. The urine was collected during morning hours directly into a sterile dipper and then immediately transferred into a sterile plastic container and stored at 2-5°C for a maximum of one day.

Detection of the exact stage of the oestrous cycle was achieved by clinical examination and laboratory tests. During clinical examination, the presence and character of the vaginal discharge, as well as the presence and quality of vulvar oedema and tolerance reflex, were evaluated. Laboratory tests consisted of vaginal cytology and analysis of progesterone concentration in peripheral blood (Kustritz 2005, 2006). Progesterone concentration was determined by commercial radioimmunoassays validated for dog blood plasma (Progesterone Coat-a-Count kit, Diagnostic Products Corporation, Los Angeles, CA, USA) (Srikandakumar et al. 1986). Eight ml of blood were taken by venipuncture from the cephalic vein into heparinised tubes. Plasma was separated 10 min after blood collection by centrifugation for 15 min at 2000 x g. Progesterone concentration was determined on the same day using the afore-mentioned RIA method.

Sample preparation

An individual standard solution was prepared by dissolving 10 mg of methyl paraben in 10 ml of methanol. Then, a series of five working standard solutions ranging in concentration from 0.005 to 5 μg/ml of methyl paraben were prepared. Solid-phase microextraction (SPME) was used to extract methyl paraben from each standard prior to GC-MS analysis. An appropriate volume (1-10 μl) of methyl paraben solution was added to 10 mL of water with 5 mL of dichloromethane and shaken for 2 min. After centrifugation, the organic phase was collected, dried over anhydrous sodium sulphate and condensed in a stream of nitrogen (Canosa et al. 2006). The residue was exposed to SPME fiber (Divinylbenzene/Car-

boxen/Polydimethylsiloxane) for about 20 min at 60°C. After exposure for 20 min, the SPME fiber was retracted into the needle of the holding syringe, solvent drops attached to the metallic needle were removed using a paper tissue and the fiber was exposed to GC-MS for 5 min (Fei et al. 2011). The capability of the equipment used in the experiment to detect methyl paraben was confirmed during a preliminary study, when MP was added to samples of urine obtained from bitches in anoestrus. Because during this part of the study it was also confirmed that the results of detecting methyl paraben in water and in urine were identical, in the experiment water was used as a matrix. Similar approaches were applied also by other researchers (Goodwin et al. 2008, Arnaiz et al. 2014). Risticevic et al. (2010) also used this SPME method for identification of MP in a much more complex matrix which is sawage water.

Chromatographic conditions

The chemical composition of the volatile compounds absorbed on the fiber was analysed using a gas chromatograph (GC) coupled to a mass spectrometer (MS), using a Saturn 2000 MS Varian Chrompack with a ZB-1 (Phenomenex) column (30 m x 0.25 µm film x 0.25 mm ID). The MS was equipped with an ion-trap analyzer set at 1508 for all analyses with an electron multiplier voltage of 1350 V. Scanning (1 scan s^{-1}) was performed in the range of 39-400 m/z using electron impact ionization at 70 eV. The analyses were carried out using helium as the carrier gas at a flow rate of 1.0 mL min^{-1} in a split ratio of 1:20 and the following program: 60°C at the beginning and holding for 3 min; 3°C/min up to 120°C; then 15°C/min to 300°C. The injector and detector were held at 200 and 300°C, respectively. Analyses were carried out using helium as the carrier gas at a ow rate of 1.0 mL min^{-1}, in splitless mode in SPME and liquid injection mode.

The compound was identified by using three analytical methods: Kovac indices (KI), GC/MS retention times of authentic chemical-standards (S) and mass spectra of compounds and NST05 spectral library collection (MS). The retention index standards used in this study consisted of a mixture of aliphatic hydrocarbons ranging from C-5 through C-17 dissolved in methanol.

Results

For the SPME as well as the liquid injection mode of MP detection in urine, the limit of detection (LOD) and limit of quantification (LOQ) were determined. The initial parameters were established from the signal-to-noise ratios of 3 and 10, respectively. In the case of SPME, LOD was found to be 0.005 µg/ml for methyl paraben, while liquid injection gave a LOD of 0.05 g/mL.

The SPME approach for MP analysis gave satisfactory results. The LOD level was lower than that reported by Lee et al. (2005) of 0.01 µg/ml (defined for a signal-to-noise ratio of 3) using solid-phase extraction, solvent evaporation and derivatization of the analyses with pentafluoropropionic anhydride. Determination was performed using stationary phase C8 with wavelength 254 nm, with separation using a mobile phase of methanol: water (60:40 w/w). The LOD level after optimization of the HPLC method was determined at 0.035 µg/ml (Imamović et al. 2012). In comparison to other methods for the determination of MP in cosmetics, LODs were higher than those obtained using the SPME method. In all our samples tested, in liquid injection mode as well as SPME we did not find any traces of MP.

Discussion

Semiochemical signalling is one of the oldest means of communication used by organisms of all taxa. Even though studies dedicated to identification of the active biological compounds in animal secretions have been performed for many years in laboratories all over the word, the results and consequently our understanding of this primitive communication method seem to be still not fully satisfactory (Pageat and Gaulnier 2003).

Analyzing the issue of canine sex pheromones, we can clearly see that the publication by Goodwin at al. (1979) is still the most often cited study (Person 1985, Pageat and Gaulnier 2003, Kustritz 2005, Santos et al. 2013, Wani et al. 2013). Described as a primary dog sex pheromone, methyl paraben has become a component of commercially available products (Eau D'Estrus, Synbiotics, USA) recommended to both veterinarians and breeders as a useful tool for male canine sexual stimulation (Kustritz 2005, Kutzler 2005). However, analysis of the literature on the activity of methyl paraben in the context of sexual arousal shows a lack of efficiency of this substance (Kruse and Howard 1983, Tonosaki and Tucker 1985, Dzięcioł et al. 2011). In the original article reporting an effective association between PGF2h and methyl 4-hydroxybenzoate prior to electroejaculation in dogs, while the authors obtained better results in a group of males exposed to methyl paraben, they also confirmed that there were no signs of sexual arousal observed in

these males (Santos et al. 2013). Also, while the influence of natural pheromones on physiological parameters (*e.g.*, heart rate) in dogs was previously described by Dzięcioł et al. (2012b), Santos et al. (2013) did not find any differences between groups in heart and respiratory rate or rectal temperature. In this case, a probable explanation for the better results obtained in males injected with PGF2α and stimulated by MP is the stimulating influence of PGF2α (Estienne and Harper 2000, Kozink et al. 2002).

In a report by Goodwin et al (1979), except the methyl paraben other substances like diethyl phthalate (DEP) $C_{12}H_{14}O_4$, dibutyl phthalate (DBP) and heptadecane $C_{17}H_{36}$ were identified as compounds present in canine urine. All these substances could be easily classified as contaminants of the samples: for example dibutyl phthalate (DBP) is a commonly used plasticizer also used as an ectoparasiticide, and diethyl phthalate (DEP) $C_{12}H_{14}O_4$ can be transferred from plastics (also plasticizers) and is often used in cosmetics and fragrances. It is worth noting that methyl paraben ($CH_3(C_6H_4(OH)COO$) (CAS No. 99-76-3) (E218) is also very often present in our environment. It is a stable, non-volatile compound and except for natural sources (fruits) it has been commonly used as an antimicrobial preservative in foods, drugs and cosmetics for over 50 years (Soni et al. 2002). While the influence of MP on the sexual behavior of dogs has been questioned by many authors, its accidental presence in evaluated samples, similar to other chemicals mentioned above, appears to be highly probable.

Taking into account results obtained during our study and emerging doubts after reading reports describing compounds in the urine of bitches, we conclude that methyl p-xydroxybenzoate cannot be considered as the main canine sex pheromone because it is not a normal component of canine urine. This conclusion is in agreement with our previous results presenting the lack of efficiency of commercially available products containing methyl paraben in creating sexual arousal in male dogs (Dziecioł et al. 2011). Thus, the question of identification of the canine sex pheromones is still open and the need for further detailed studies is indicated.

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