CHEMICAL CHARACTERIZATION OF BOVINE (*Bos taurus*)
URINE DURING PREGNANCY

(CARACTERIZAÇÃO QUÍMICA DA URINA DE BOVINOS (*Bos taurus*)
DURANTE A PRENHEZ)

K. RAMESH KUMAR¹, G. ARCHUNAN¹, ²

SUMMARY

Gas chromatography linked mass spectroscopy (GC-MS) was used to examine the chemical profiles from early, mid and late pregnant bovine urine and were compared qualitatively. Results indicate that fourteen different volatile compounds were identified in urine, which include alcohols, aldehydes, ketones, alkanes and alkenes. The chemical profiles of pregnant urine are entirely different from that of estrus urine. The compounds identified in one phase do not correlate with other phases during gestation period. The variation in the chemical profile indicates that the excreted urine are not uniform throughout the pregnancy and appears to conclude that the difference of chemical compounds present in the pregnant cow urine may depend upon the physiological state of the female.

KEY-WORDS: Bovine, chemical characterization, pregnancy, urine, pheromone, gas chromatography linked mass spectrometry

RESUMO

A cromatografia a gás acoplada à espectrofotometria de massa (CG-MS) foi utilizada para verificar as propriedades químicas nos terços inicial, médio e final da gestação em animais da espécie bovina. As mensurações foram comparadas qualitativamente. Os resultados indicaram a presença de quatorze compostos voláteis, entre eles, álcoois, aldeídos, cetonas, alcanos e alcenos. As características químicas da urina de animal prenhe são totalmente diferentes quando comparadas às encontradas na fase de estro. Os compostos identificados no terço inicial não se correlacionam com aqueles das outras fases subsequentes do período gestacional. A variação da característica química indicou que a composição da urina excretada não é uniforme em todo o período de gestação. Portanto, pode-se especular que a composição da urina, durante o período gestacional, pode depender do estado fisiológico da fêmea.

PALAVRAS-CHAVE: Bovino, caracterização química, gestação, urina, feromônio, cromatografia a gás acoplada à espectrofotometria de massa.

1 Department of Animal Science, Bharathidasan University, Tiruchirappalli - 620 024, Tamil Nadu, India. Phone - 660360, Fax 660245 e-mail:archu@bdu.ernet.in and krames@bdu.ernet.in
2 Contact author
INTRODUCTION

The role of chemical signals in reproductive behavior has been clearly established in several species of mammals. Urine, faeces and vaginal contents, as well as exocrine glandular products, function as sex attractants and serve to facilitate the location and recognition of mates in mammals (ARON 1979; BALAKRISHNAN & ALEXANDER 1985; DOMINIC 1991; JOHNSTON et al., 1993). Studies on the properties of pheromones have been conducted mainly on laboratory animals such as mouse, rat and rabbit, and at the same time considerable works have also been reported on farm animals (KLEMM et al., 1987; SIGNORET, 1991), as well as in human (MCCLINTOCK, 1983). The fact that the bull detects the cow estrus is one of the striking example for olfactory communication (KLEMM et al., 1987). It has been drawn much attention that the urinary marking fluid of tigers may be used as fingerprinting for individual identification and it gives additional support for the importance of urinary compounds for individual recognition (PODDER-SARKAR & BRAHMACHARY, 1999).

The chemical identity of mammalian urinary pheromones from mouse (NOVOTNY et al., 1985), rat (SELVARAJ & ARCHUNAN, 2000), tiger (BRAHMACHARY et al., 1991), elephant (RASMUSSEN et al., 1996) and bovine (RAMESH KUMAR et al., 2000) is available. The volatile compounds of mammalian urinary samples have revealed a number of compounds like alcohols, aldehydes, ester and ketone (RASMUSSEN & LEE, 1991). Furthermore phenol and dimethylsulfone have been reported to be the major constituents of the human urine (WILLIAMS et al., 1966).

Our recent study revealed that seven different volatile compounds are found in bovine urine during estrus cycle. Of those, the compounds 1-ioudondecane and di-n-propylphthalate are particularly present in ovulatory phase which are exclusively absent in preovulatory and postovulatory phases. Behavioral assay convincingly demonstrated that compounds act as sex attractants towards bull (RAMESH-KUMAR, 2000). It has become evident that excretory products either in the form of urine or faeces serve as purposeful communicators. It is necessary to confirm whether the compounds identified in ovulatory phase are present in pregnant urine. To date no chemical investigations have been performed in the pregnant cow urine. Therefore, the present investigation was undertaken to compare the chemical profiles of pregnant cow urinary volatiles with the estrus volatile compounds so as to confirm whether the pregnant urine contains any of the volatiles found in estrus urine. This study represents our initial step in the investigation of urinary compounds that are critically involved in the olfactory communication.

MATERIAL AND METHODS

The pregnant urine of three different periods were collected from the healthy animals at the Exotic Cattle Breeding Centre, Tanjore, Tamil Nadu, India. Twenty healthy pregnant cows (Jersey) fed on a standard diet were used. Four urine samples were collected from all the three different periods, i.e. early (1-3 months), mid (4-6 months), and late (7-9 months) gestation. Urination was induced by manual stimulation of the perineal region. The excreted urine (100 mL) were screened through cheese cloth or nylon mesh (60 - 120 m) and was put into a clear glass jar at each collection. Immediately after collection, samples were stored frozen at -20°C and analyzed within a week.

The samples collected from the various cows in a particular stage were pooled in order to avoid individual variations. For the extraction of the compounds from the pooled urine nine different organic solvents such as acetone, benzene, chloroform, dichloromethane, diethyl ether, ethanol, n-hexane and petroleum ether were used. Among those, dichloromethane (DCM) extraction showed maximum response, therefore, the DCM was used as a solvent in the present study. Each sample was thawed at room temperature prior to analysis. Then triplicate 1 mL of samples of the pooled urine were taken and separately mixed with 1 mL of dichloromethane. The supernatant was filtered through a silica gel column (60-120 mesh) and concentrated under vacuum (temp. <30°C) for fractionation and chemical identification by gas chromatography linked mass spectroscopy (GC-MS) analysis. The GC-MS analyses were made in QP-5000, (Shimadzu, Japan). The 2 mL of extract was injected into the GC-MS on a 30 m glass capillary column with a film thickness of 0.2 mm (30 m X 0.2 mm i.d. coated with UCON HB 2000) using the following temperature programme, initial oven temperature of 40°C for 4 minutes increasing to 250°C at a rate of 15°C for 10 minutes. The GC-MS was also under computer control a 70-ev. Chemical ionization was performed by using ammonia as reagent gas at 95-ev. Identification of unknown compounds was made by probability based matching using the computer library built within the NICT 12 system.

RESULTS AND DISCUSSION

The GC are the representative of the urinary compounds obtained in the three periods viz. early, mid and late gestation. Nearly 14 compounds were identified.
as major constituents in the pregnant urine (Fig. 1), and
some differences were also noted when comparing the GC-
MS profiles of three periods (Table 1). Notably the
compounds found in one period are not found in other two
periods. The compounds found in three periods of pregnant
urine are as follows: viz. 1-phenol, 4, (2-aminopropyl),
propane, n-hexylmethylamine, undecane, tridecane,
2,9, dimethyldecane in early gestation; 1-nitropentane, 1-
chloro-2-heptene, 2-cyclopenten-1-one-5-hydroxy-2-3
dimethyl in mid gestation and 5,6 diamino-2,4
dihydropryrimidine, 1,1 dichloro-2-nonene (E), tetradecane,
p-mentha-6,8 dien-2-one semicarbazone, 1,4 benzene
dicarboxylic acid in late gestation. The structure of the
identified compounds is given in Fig. 2.

Urine is an accurate indicator of the physiological
status of an animal because of its function as the major
means by which metabolic waste is eliminated from an
animal (ALBONE, 1984). The chemical compounds
present in one period are not appeared in the other stages.
This indicates that the chemical compounds excreted in
the urine are not uniform throughout the pregnancy. As
many urinary compounds are also found in the blood, it is
not surprising that urine can convey to the external
world much information concerning the internal
physiological state of the animal, and so provide a
source of chemical signals in many species. The

Table 1 - Urinary compounds found in pregnant bovine (Bos taurus) urine. (+) indicates the presence of compounds.
(-) indicates the absence of compounds.

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Compound Class</th>
<th>Name of the compounds</th>
<th>Mol. Wt.</th>
<th>Mol. For.</th>
<th>EP</th>
<th>MP</th>
<th>LP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Phenol</td>
<td>1-phenol, 4 (2-aminopropyl)</td>
<td>151</td>
<td>C8H13NO</td>
<td>+</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>2</td>
<td>Alkane</td>
<td>Propane</td>
<td>44</td>
<td>C3H8</td>
<td>+</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>3</td>
<td>Alkylamine</td>
<td>n-hexylmethylamine</td>
<td>115</td>
<td>C7H17N</td>
<td>+</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>4</td>
<td>Alkane</td>
<td>Undecane</td>
<td>156</td>
<td>C11H24</td>
<td>+</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>5</td>
<td>Alkane</td>
<td>Tridecane</td>
<td>184</td>
<td>C13H28</td>
<td>+</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>6</td>
<td>Alkane</td>
<td>2,9-dimethyldecane</td>
<td>179</td>
<td>C7H13Cl</td>
<td>---+</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>7</td>
<td>Nitroalkane</td>
<td>1-nitropentane</td>
<td>117</td>
<td>C7H6O2</td>
<td>---+</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>8</td>
<td>Haloalkene</td>
<td>1-chloro-2-heptene</td>
<td>132</td>
<td>C6H10N4</td>
<td>---+</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>9</td>
<td>Cycloalkene-alcohol</td>
<td>2-cyclopenten-lone, 5-hydroxy-2,3-dimethyl</td>
<td>126</td>
<td>C6H10N4</td>
<td>---+</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>10</td>
<td>Heterocycle</td>
<td>5,6-diamino-2,4 dihydropryrimidine</td>
<td>142</td>
<td>C8H6Cl2</td>
<td>---+</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>11</td>
<td>Haloalkane</td>
<td>1,1-dichloro-2-nonene (E)</td>
<td>194</td>
<td>C14H30</td>
<td>---+</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>12</td>
<td>Alkane</td>
<td>Tetradecane</td>
<td>198</td>
<td>C11H17N3O</td>
<td>---+</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>13</td>
<td>Semicarbazone</td>
<td>p-mentha-6,8dien-z-one semicarbazone</td>
<td>207</td>
<td>C8H6O4</td>
<td>---+</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>14</td>
<td>Acid</td>
<td>1,4-benzene dicarboxylic acid</td>
<td>174</td>
<td>---+</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>
Figure 1 - GC chromatograms of the identified urinary compounds in the three stages of pregnant cows (Bos taurus).

Potential role of the urinary compounds of some laboratory rodents has been thoroughly studied, and relative effects have been established (DOMINIC, 1991; JOHNSTON et al., 1993). The present study focused on the analysis of GC-MS profiles of the volatile compounds of bovine urine in three different phases viz. early, mid and late gestation and found the urinary compounds differed qualitatively.

Comparisons of the urinary profiles between estrus and pregnant samples indicate that there are some specific volatile compounds observed in estrus which are not found in the pregnant urine. Since a natural estrous cycle is caused by hormonal changes, the above qualitative variations in the urine may also be due to endocrine changes. The endocrine dependency of some urinary volatile compounds in the mouse has been demonstrated (ANDREOLINI et al., 1987; BOYER et al., 1988; JEMILOLO et al., 1994). It is also confirmed in the present study that the compounds 1-iodoundecanae and di-n-propyphthalate identified in the estrus urine (RAMESH KUMAR et al., 2000) are not present in the pregnancy urine. It shows that the estrus phase has capable of producing the specific odour.

Several reports indicate that animals will communicate to other members of the same species through excretory sources. In the present study the bovine expresses their reproductive status to other members of community through excreting specific chemical compounds during gestation by which other members of the community will identify the status of the reproductive condition. It appears that variation in the chemical compounds during gestation may depend upon the physiological state of females. Female mice treated with urine from pregnant or lactating females experience longer periods of vaginal cornification than those treated with water or urine from normal females (HOOVER & DRICKAMER, 1979) and indicate that female even during gestation or lactation excrete varieties of signals through urine and that could modify the reproductive status of other animal. The compound, undecane, found in early gestation is already reported in postovulatory urine (RAMESH KUMAR et al., 2000) but its biological importance is not yet known. It is noteworthy that the late pregnant urine contains high molecular weight compounds as compared with early and mid pregnant urine samples. Therefore, it is reasonable to presume that the compounds present in the late gestation may use as biochemical marker as single or combined for identifying the parturition time in cow. Further study is required to confirm the biological functions of each compounds identified in the pregnant urine.

Figure 2 - Chemical structures of the identified urinary compounds of pregnant cows (Bos taurus) urine.
CONCLUSION

The compounds identified earlier in estrus urine are exclusively absent in the pregnant urine. In contrast to early and mid pregnant the late pregnant urine contains high molecular weight compounds and suggests that the compounds present in the late gestation may be used as biochemical markers for identifying the parturition time in cow. The pregnant females excrete varieties of signals throughout the pregnancy by which they convey the reproductive status to the herd mate. The present results also suggest that variation in the chemical compounds during pregnancy may depend upon the physiological state of females.

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