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# Detection and qualitative analysis of fatty acid amides in the urine of alcoholics using HPLC-QTOF-MS

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ABSTRACT

Fatty acid amides (FAAs) in alcoholism lead to liver diseases. These amides have been reported in plasma and in other organs of the body, while their detection or presence in the urine is still unknown. Therefore, the focus of the current study was to detect and analyze FAAs qualitatively in urine samples of alcoholics. Furthermore, the effects of *Tinospora cordifolia* (hepatoprotective medicinal plant) intervention on FAA levels in moderate alcoholics were also analyzed. In the study, asymptomatic chronic alcoholics ($n = 22$) without chronic liver disease and nonalcoholic healthy volunteers ($n = 24$) with a mean age of 39 ± 2.0 years were selected. The first-pass urine and fasting blood samples were collected in the morning on day 0 and day 14 after *T. cordifolia* water extract (TCE) treatment and analyzed using automated biochemistry analyzer and HPLC-QTOF-MS. Results indicated the increased levels of serum triglycerides, cholesterol, and liver function enzymes in alcoholic subjects, which were significantly down-regulated by TCE intervention. Multivariate discrimination analysis of QTOF-MS data showed increased urinary levels of oleoamide (2.55-fold), palmitamide (5.6-fold), and erucamide (1.6-fold) in alcoholics as compared to control subjects. Levels of oleamide (1.8-fold), palmitamide (1.7-fold), and linoleamide (1.5-fold) were found to be increased in plasma. Treatment with TCE in alcoholics (3.0 g lyophilized water extract/day) significantly decreased the plasma and urinary levels of all FAAs except linoleamide. The HPLC-QTOF-MS approach for FAAs analysis in both urinary and plasma samples of alcoholics worked very well. Moreover, findings (i.e., increased levels of FAAs in urine and in plasma) further support other findings that these amides play a very important role in alcoholism. Further, like our previous findings, TCE proved its hepatoprotective effect against alcoholism not only by lowering the levels of these detected FAAs, but also by decreasing the level of liver-specific enzymes and lipids.
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## Introduction

Fatty acid amides (FAAs) are bioactive lipids thought to be involved in the regulation of various physiological process, including modulation of mono-aminergic systems, feeding, sleeping, reproduction, and inflammation (Fu et al., 2003; Lo Verme et al., 2005; Piomelli, 2003). FAAs (endocannabinoids) are agonists of endocannabinoid signaling and are believed to play a role in alcohol abuse, as FAAs and alcohol consumption activate the same reward pathways (Mechoulam & Parker, 2003). Increased FAAs levels and decreased GSH levels also cause liver cell toxicity and cell death (Siegmund et al., 2006). Moreover, inhibition of FAAH (fatty acid amide hydrolase) has been reported to reduce anxiety associated with alcohol withdrawal by activation of the endocannabinoid system (Cippitelli et al., 2008). These converging data suggest the deficiency of FAAH in alcoholics, required to degrade FAAs. This hypothesis is further strengthened by the fact that chronic ethanol exposure has been reported to increase the anandamide levels in neuroblastoma cells (Basavarajappa & Hungund, 1999) and in cerebellar granular neurons (Basavarajappa, Saito, Cooper, & Hungund, 2000).

FAAs have been identified in several tissues and biological fluids, probably with distinct metabolic pathways, which need to be elucidated. The final fate of most of the metabolites is excretion, mainly through urine in the intact or derivatized form. However, to date, FAAs have not been detected in urine samples, in either form. This is due to their very low concentrations in biological fluids that make their separation and detection challenging (Gee, Groen, & Johnson, 1999). Therefore, FAAs have been detected and

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quantified using gas chromatography (GC) and high-performance liquid chromatography (HPLC), mainly in the derivatized form (Dolowy & Pyka, 2015). Further, it is necessary to extract FAAs to examine their presence and levels in biological samples, as their excretion pattern in any diseased condition is not known. Moreover, at present there is no literature available on FAAs in the urine sample. Thus, the present study was undertaken to examine levels of FAAs in unprocessed urine and plasma samples from alcohol consumers using HPLC-ESI-QTOF-MS. Simultaneously, the effects of *Tinospora cordifolia* water extract (TCE, known to be beneficial in the treatment of alcoholism) on the urinary and plasma levels of FAAs in alcohol consumers were also analyzed (Mittal & Dabur, 2015; Sharma & Dabur, 2016).

## Materials and methods

### *Chemicals*

Water, acetonitrile, formic acid, calibrants, and internal standards (oleamide, erucamide, palmitamide, and linoleamide), all of mass spectroscopy grades, were purchased from Sigma Aldrich (St. Louis, MO). All the solvents used in the study were of LC-MS grade. Calibrants and standards for high performance liquid chromatography-quadrupole time of flight mass spectrophotometer (HPLC-Q-TOF-MS) were purchased from Agilent Technologies (Santa Clara, CA, USA).

### *Drug preparation*

Water extract (TCE) was prepared from fresh stems of *T. cordifolia* cultivar collected from the garden of the National Research Institute of Basic Ayurvedic Sciences, Nehru Garden, Kothrud, Pune, India in September 2011. The voucher specimen (No. 207) was deposited in the medicinal plant museum of the Institute. The stems were washed with distilled water, crushed, and kept in reverse-osmosis water (20:100 w:v of the stem and water) overnight at room temperature. The next morning, the water extract was filtered. One hundred mL of the fresh extract (3.0 g after lyophilization) was given to alcoholic subjects under the guidance of an Ayurvedic physician to avoid degradation, as reported earlier (Shirolkar, Gahlaut, Hooda, & Dabur, 2013).

### *Study subjects*

The Human Ethics Committee of the DYP Ayurveda College, Pune, India approved the study-wide letter no. RRI/2011/HEC/2023 dated November 18, 2011. Volunteers were selected based on their drinking status, gender, and age, and underwent the questionnaire of alcohol-use disorders identification test (AUDIT). All volunteers signed an informed written consent. Male alcoholics ($n = 22$) and non-alcoholic male ($n = 24$) volunteers of mean age 39 ± 2 years, having a mean body mass of 62.3 ± 2.2 (mean + SD) kg were selected out of 230 volunteers. Only the volunteers regularly drinking 60–80 mL of 80-proof liquor (40% alcohol; 24–36 g of alcohol) per day during the previous year and at least 6 days in a week were included in the study. Clinical and demographic data were collected at the time of enrollment (Table 1). No volunteers reported any disease or the use of any kind of regular medication. Each individual entered into the study signed an informed written consent. Exclusion criteria included body mass index >30 kg/m$^2$, blood pressure >160/90 mm Hg, total cholesterol >7.5 mmol/L, present or prior history of cardiovascular disease, diabetes mellitus, respiratory, gastrointestinal, hepatic, renal, endocrine, or reproductive disorders, or use of lipid-lowering agents or antihypertensive agents.

**Table 1**
Demographic information, liver function, and lipid profile of the study population.

| Variables | Control group ($n = 24$) | Alcoholics ($n = 22$) | TCE-treated alcoholics ($n = 20$) | $p$ value |
|---|---|---|---|---|
| Sex | Male | Male | Male | na[b] |
| Age | 39 ± 1.8 | 39 ± 2.1 | 39 ± 2.1 | ns[a] |
| BMI | 23.67 ± 0.22 | 23.1 ± 0.27 | 23.47 ± 0.21 | ns[a] |
| AST (U/L) | 20.50 ± 5.2 | 85.6 ± 6.4 | 38.6 ± 4.2 | <0.05 |
| ALT (U/L) | 22.1 ± 4.4 | 53.8 ± 6.8 | 29.8 ± 5.6 | <0.05 |
| GSH (mmol/L) | 1.5 ± 0.2 | 0.85 ± 0.3 | 1.2 ± 0.2 | ≤0.05 |
| MDA (mmol/L) | 3.3 ± 0.7 | 9.7 ± 1.4 | 4.2 ± 0.5 | ≤0.05 |
| γ−GT (U/L) | 10.8 ± 4.1 | 68.5 ± 8.2 | 38.7 ± 6.3 | <0.05 |
| TC (mg/dL) | 150.3 ± 11.2 | 211.5 ± 9.7 | 173.7 ± 9.4 | <0.05 |
| TG (mg/dL) | 91.5 ± 7.7 | 166.4 ± 9.6 | 109.7 ± 7.7 | <0.05 |
| MCV (fL) | 86.3 ± 0.6 | 96.7 ± 0.5 | 92.9 ± 0.6 | <0.05 |

Values for hematology and biochemistry were reported as mean ± standard error. Student's $t$ test for the unpaired data was used to determine the statistical significance. The difference was considered as significant at a $p$ value of ≤0.05.
[a] ns = not significant.
[b] na = not applicable.

### *Sample collection and processing*

Morning first-pass urine and fasting blood samples were collected on day 0 and day 14. Blood samples were collected into EDTA and reduced-glutathione containing tubes. The samples were subjected to centrifugation at 1000 × $g$ for 10 min at 4 °C. To overcome the oxidation of samples, butylated hydroxytoluene (40 μg/mL plasma) was added to the plasma samples, which were stored at −80 °C until analysis.

### *Extraction and analysis of FFA in plasma and urine samples*

For FAAs analysis, 100-μL plasma samples were mixed with acetone and acetonitrile in a 1:1 ratio and stored at −80 °C overnight to precipitate the proteins. Afterward, samples were centrifuged for 10 min at 14,000 × $g$ at 4 °C. Resultant supernatants containing FFAs were separated, lyophilized, and stored at −80 °C for further use. For LC-MS analysis, each sample was reconstituted in 200 μL of acetonitrile and filtered through a 0.2-micron membrane filter. The extraction process of FFA from a urine sample is very similar to the extraction process from plasma except for the protein precipitation step. For urine samples, instead of using a mixture of acetone and acetonitrile, we used only 200 μL of acetonitrile for 100 μL of urine for precipitation of proteins and extraction of metabolites.

### *Liquid chromatography*

Urine and plasma samples were resolved over a Poroshell 120-SB C18 column (3 × 100 mm, 2.7-μm particle size) fitted with an HPLC system-consisting binary pump with autosampler, and coupled with Q-TOF-MS (Agilent 1290 Infinity Series HPLC). The solvent system had (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile:water (80:20). The flow rate was 0.2 mL/min. The linear gradient mode was optimized as follows: 0–18 min, 20–50% B; 19–30 min, 50–90% B; 30–40 min, 90% B; 41–45 min, 20% B; 46–50 min, 20% B.

### *Mass spectrometry*

Q-TOF-MS (Agilent 6538 Accurate-Mass Q-TOF-MS) was operated in the positive ion polarity and ESI mode with acquisition rate 3 spectra/s. The collision cell energy and collision cell flow were maintained at 15–37 V and 22 psi. Voltages of fragmenter 1, 2, and capillary (Vcap) were maintained at 210, 195, and 3500 V,

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