Sánchez-López, Elena et al., 2017. Investigation on the combined effect of cocaine and ethanol administration through a liquid chromatography–mass spectrometry metabolomics approach. Journal of pharmaceutical and biomedical analysis, 140, pp.313–321.

Available at https://doi.org/10.1016/j.jpba.2017.03.061

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PII: S0731-7085(16)31267-5
DOI: http://dx.doi.org/doi:10.1016/j.jpba.2017.03.061
Reference: PBA 11189

To appear in: *Journal of Pharmaceutical and Biomedical Analysis*

Received date: 30-11-2016
Revised date: 27-3-2017
Accepted date: 29-3-2017

Please cite this article as: Elena Sánchez-López, Alberto Marcos, Emilio Ambrosio, Oleg A.Mayboroda, María Luisa Marina, Antonio L.Crego, Investigation On The Combined Effect Of Cocaine And Ethanol Administration Through A Liquid Chromatography-mass Spectrometry Metabolomics Approach, Journal of Pharmaceutical and Biomedical Analysishttp://dx.doi.org/10.1016/j.jpba.2017.03.061

INVESTIGATION ON THE COMBINED EFFECT OF COCAINE AND ETHANOL ADMINISTRATION THROUGH A LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY METABOLOMICS APPROACH

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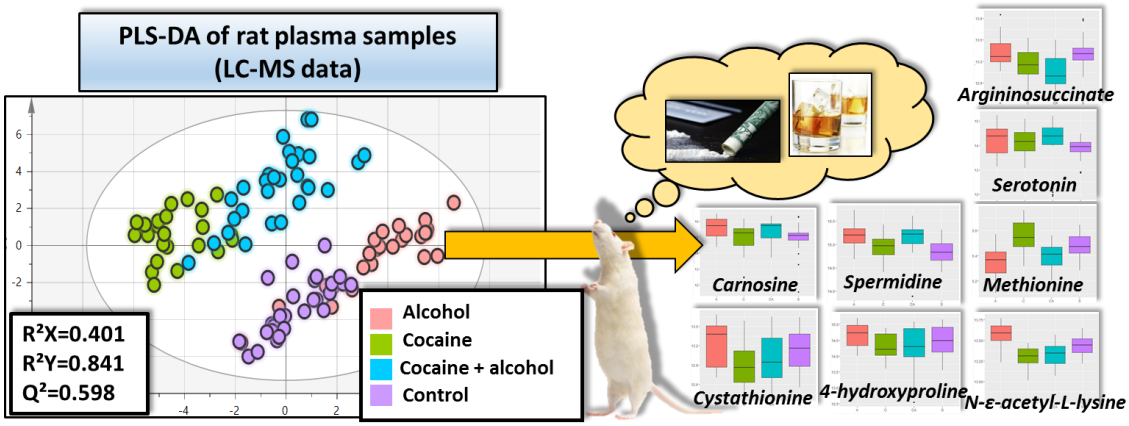
Graphical abstract:



Highlights:

- First metabolomics study on effects of combined cocaine and alcohol.
- Metabolic profiling approach based on LC-MS analysis of rat plasma samples.
- Derivatization enabled polar compounds to be retained on a C18 column.
- Multivariate analysis showed good classification of experimental groups.
- Some metabolic pathways affected include Trp, Arg, Pro, Met, and Cys metabolism.

Abstract

Alcohol is the most widely consumed legal drug, whereas cocaine is the illicit psychostimulant most commonly used in Europe. The combined use of alcohol and cocaine is frequent among drug-abuse consumers and leads to further exacerbation of health consequences compared to individual consumption. The pharmacokinetic and metabolic interactions leading to an increase in their combined toxicity still remains poorly understood. Here, the first metabolomics study of combined cocaine and ethanol chronic exposure effects is reported. A Liquid Chromatography strategy based on sample derivatization with 9-fluorenylmethyloxycarbonyl chloride and using a C18 column coupled to high resolution Mass Spectrometry (time of flight analyzer) was employed to

analyze plasma from rats exposed intravenously to these drugs in a 52-min analysis. Using a combination of non-supervised and supervised multivariate analysis ~~we~~ the metabolic differences between our experimental groups were explored and unraveled. A comparative analysis of the individual models and their variable importance in the projection values have shown that every experiment intervention includes a subset of specific metabolites. Eleven of these metabolites were annotated, where eight were unequivocally identified using standards and three were tentatively identified by matching the MS/MS spectra to libraries. The results demonstrated that the affected metabolic pathways were mainly those related to the metabolism of different amino acids.

Keywords: cocaine / drug dependence / ethanol / metabolomics / liquid chromatography - mass spectrometry / rat plasma

1. Introduction

Cocaine is the illicit psychostimulant drug most commonly used in Europe . It is well known that it produces changes in several monoaminergic neurotransmitter systems and has deleterious effects on several metabolic pathways. In Europe a high percentage of cocaine users combine cocaine with ethanol intake . When used in a combined way, alcohol consumption increases the plasma levels of cocaine [1]. Moreover, it is known that alcohol modifies the biotransformation of cocaine, both in rats [2] and in humans [3]. A combination of alcohol and cocaine leads to the formation of a psychoactive metabolite, cocaethylene known for its toxicity and ability to increase the blood pressure and the heart rate [1]. This is of special concern during early stages of life, since exposure to alcohol or other drugs can affect the development of the neurological system leading to neuropsychological pathologies during later stages of development [4]. For instance, in

Spain, the average age when young people begin to consume alcohol is 16 years whereas for cocaine the average "starting age" is 21 years [5]. In the 15-24 age bracket, the prevalence of alcohol use is 83% in Italy and France and 89% in Germany. The prevalence of cocaine use is 0.9%, 1%, and 2.6% in Italy, Germany, and France, respectively [6]. The effect of combined consumption of cocaine and alcohol has been scarcely studied despite high prevalence and socioeconomic relevance of the problem.

Metabolomics is a postgenomic discipline enabling a global, unbiased overview of the physiological/biochemical effects of drug intake [7]. Metabolomics enables to capture the effect of an external stimulus on the endogenous metabolome, understood as the totality of small molecules which are involved in different metabolic pathways required for the maintenance, growth, and normal function of a cell [7]. By comparing samples between a normal state and a perturbed state, differences in the levels of metabolites that are a direct consequence of the stimulus can be highlighted and can be subsequently mapped to specific metabolic pathways and thus provide with a better understanding of the effects of such stimulus [7]. Contemporary metabolomics has a broad analytical base which includes techniques such as Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS). The last one is the most widely used one, due to its high sensitivity and wide coverage [8]. MS can be easily coupled to the separation techniques such as Liquid Chromatography (LC), Gas Chromatography (GC), or Capillary Electrophoresis, with LC-MS being the most popular one.

Although the consequences of consumption of either cocaine or alcohol individually have been investigated by metabolomics studies, to the best of our knowledge, combined chronic consumption of cocaine and alcohol has never been explored using such approach. Metabolomics studies of subjects treated only with cocaine have been reported by different analytical techniques, these being NMR [9], ambient

pressure ion mobility mass spectrometry [10], LC with electrochemical detection [11], and GC-MS [12]. As far as we know, LC-MS has never been used to investigate the effects of this psychostimulant drug. On the other hand, effects of the administration of only ethanol have been reported in some metabolomics contributions, which have been gathered in a review article [13]. From that review it can be summarized that the most used platform for this kind of studies has been LC-MS employing reversed-phase stationary phases (RPLC-MS). As authors there remark, there is a need to evaluate how hydrophilic compounds behave in these studies, given that there is a huge variety of important biological molecules which are hydrophilic (e.g. amino acids and amines) [13]. Recently, our research group developed a RPLC-MS methodology which includes a derivatization step with the labelling reagent 9-fluorenylmethyloxycarbonyl chloride (FMOC-Cl), known for its ability to form stable compounds rapidly with primary and secondary amines, so that hydrophilic compounds can be retained and separated in a standard C18 column [14].

In this work, the effects of combined chronic consumption of cocaine and alcohol on the metabolic profiles of blood plasma was investigated. Rats which were 51 ± 2 days old upon initiation of treatment were used. At this age, the rats enter into the "young adult" phase [15], corresponding roughly in humans to when consumption of both drugs has already been initiated. The objective of this work is to find out if differences between administration of cocaine or alcohol and their combined administration can be established through a metabolomics approach. If possible, those differentiating metabolites will be identified and allocated in the different metabolic pathways.

2. Materials and methods

2.1. Reagents

All reagents employed in this work were of analytical grade. MS-grade acetonitrile and methanol were obtained from Scharlau Chemie (Barcelona, Spain). Boric acid, formic acid, ammonium carbonate, ammonium hydroxide, sodium hydroxide, pentane, and FMOC were from Sigma (St. Louis, MO, USA). Isoflurane was from Abbott (Madrid, Spain). Ethanol at 96% (v/v), suitable for use as an excipient (Emprove® exp) was acquired in Sigma Aldrich (Germany). Cocaine hydrochloride was kindly provided by the Spanish Agency for Medicines and Health Products of the Ministry of Health, Social Services and Equality. Sodium chloride solution at 0.9 % (v/v) was from Laboratorios ERN (Barcelona, Spain).

Standards employed in the identification of the significant metabolites are described hereafter. Argininosuccinic acid, cytosine, cis-4-hydroxy-D-proline, trans-4-hydroxy-L-proline, N-acetyl-L-alanine, N-α-acetyl-L-lysine, 5-aminovaleric acid, L-homoserine, 2-aminoisobutyric acid, serotonin, methionine, 1-methyl-L-tryptophan, 5-hydroxy-L-tryptophan, 4-acetamidobutanoate, 5-amino-levulinic acid, N-ε-acetyl-L-lysine, 2-methylserine, isoglutamate, N-methyl-D-aspartic acid, *allo*-isoleucine, isoleucine, leucine, norleucine, valine, norvaline, threonine, arginine, γ-aminobutyric acid, N-acetylserotonin, cystathionine, and spermidine were acquired from Sigma (St. Louis, MO, USA). L-carnosine was from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Isobutyrylglycine, N-butyrylglycine, 4-methylaminobutyrate, and N-acetylserine were from Cymyt Química S. L. (Barcelona, Spain).

2.2. Animals and drug administration

Thirty-seven male and female Wistar rats were bred in-house (parents were from Charles River Laboratories, Lyon, France). The offsprings were weaned on the 21st day and were housed in monosexual groups with a 12 hours light/12 hours dark cycle (lights were turned on at 8.00 am) with food *ad libitum* (Scientific Animal Food, Augy, France).

All procedures were performed according to the animal welfare directive (Directive 2010/63/EU) and had previously been approved by the Bioethics Committee of the National University of Distance Education (Madrid, Spain).

In order to enable the intravenous administration of drugs, a polyvinyl chloride catheter (0.064" internal diameter) was implanted in the right jugular vein at the age of 43 ± 2 days, by surgery under isoflurane anesthesia by inhalation (La Bouvet, France). One day before the start and before the end of the treatment, the functionality of the catheters was tested by infusing sodium thiopental (10 mg/kg). Subjects which lost consciousness were considered to have a working catheter pathway. The treatments begun at the age of 51 ± 2 days in balanced groups. The rats were randomly assigned to a treatment group. Parity was maintained between the number of males and females. During 21 days, in 105-min daily sessions (30 infusions of 30 s per session) rats received one of the following four treatments: (i) cocaine (15 mg/kg), (ii) alcohol (2 g/kg), (iii) cocaine + alcohol (15 mg/kg and 2 g/kg, respectively), and (iv) 0.9 % (w/v) sodium chloride solution (control group). Drugs were solved in same media as the saline group (0.9 % (w/v) sodium chloride). The dose for each animal was individualized according to their weight and it was recalculated every three days. The automatic dispensing equipment consisted of a pump (Harvard 22, Harvard Apparatus, Holliston, MA, USA) controlled by a software designed *ad hoc* (Medline Industries Inc., CA, USA).

2.3. Sample collection and preparation

Rats were decapitated, and blood was recollected in heparinized tubes, centrifuged for 10 min at 2000 x g (4 ºC) and plasma was collected and stored at −70°C until sample treatment. From each of the 37 plasma samples, three individual aliquots were taken and they underwent the same sample treatment independently, so that a total of 111 plasma samples were to be analyzed.

The sample preparation is the one previously developed in our group ~~[19]~~ [14]. Briefly, a mix of 400 µL of methanol and 400 µL of 400 mM sodium borate (pH 9.0) (1:1, v/v) was added to 800 µL of plasma (1:1, v/v), then it was vortexed for 30 s and it was left still for 10 min in an ice bath. After extraction, samples were centrifuged at 10000x*g* for 15 min at 4 ºC. The supernatant was vortexed for 30 s, and was subdivided in three aliquots of 400 µL to be then ultra-filtered in three independent 3 kDa cut-off filters (Amicon Ultra Filters, Merck, Darmstadt, Germany) at 14000x*g* for 25 min at 4 ºC. 100 µL of each of the three filtrates were mixed individually with 100 µL of 20 mM FMOC in acetonitrile. The three replicates of the sample were then individually vortexed for 2 min and 200 µL of pentane was added to eliminate the excess of labelling reagent. After mixing vigorously, samples were left still for a minute and the lower fractions were taken to be analyzed.

2.4. Liquid Chromatography – Mass Spectrometry

Experiments were carried out using an 1100 series LC system (Agilent Technologies, Germany) coupled to a 6530 series quadrupole time-of-flight (QTOF) mass spectrometer (Agilent Technologies, Germany) by means of a Jet Stream orthogonal electrospray ionization (ESI) source. The LC system consisted of a degasser, a quaternary pump, an automatic injector, and a thermostatic column compartment. Agilent Mass Hunter Qualitative Analysis software (B.07.00) was used for MS control, and data acquisition. Along all analysis, two reference masses were used, *m/z* 121.0509 ($C_5H_4N_4$) and *m/z* 922.0098 ($C_{18}H_{18}O_6N_3P_3F_{24}$) for positive ionization mode, which were continually infused into the system to allow mass correction.

Experiments were carried out in a C18 Ascentis Express (Sigma, St. Louis, USA), having 100 x 2.1 mm i.d. dimensions (fused-core® particles with 0.5 µm thick porous shell and an overall particle size of 2.7 µm). In addition, a guard column (5 x 2.1 mm i.d.)

of the same composition as the analytical column was also employed. Column was kept at 40 ºC during the analytical sequence. The system operated with an injected volume of 10 μL and a flow rate of 0.4 mL/min.

Mobile phases were water (eluent A) and acetonitrile (eluent B), both with 0.1% formic acid. The linear gradient was set from 5% B to 100% B in 30 min, 100% B for 5 min and returned to starting conditions in 1 min, keeping the re-equilibration at 5% B for 15 min. The ionization source conditions were as follows: capillary voltage of 3000 V with a nozzle voltage of 0 V; nebulizer pressure at 35 psig; sheath gas of jet stream of 6.5 L/min at 275 ºC; and drying gas of 10 L/min at 275 ºC. The fragmentator (cone voltage after capillary) was set at 175 V. The skimmer and octapole voltages were 60 V at 750 V, respectively. MS analysis were performed in positive ESI mode with mass range set at m/z 70-1600 (extended dynamic range) in full scan resolution mode at a scan rate of 2 scans per second. MS/MS analyses were carried out by selecting the precursor ion at the corresponding retention time with a collision energy of 20 V using nitrogen as collision gas.

2.5. Metabolic profiling

At the beginning of the sequence several blanks and QCs were ran to ensure good repeatability in the system. Samples were randomized and a QC was injected every six samples. It is a common practice to inject the same QC pool along the sequence. However, this does not show the real method variability as, for instance, sample preparation variation is not included. Therefore, in this work, once the same aliquot was taken from all the samples and was mixed, it was divided in three portions and they underwent the same sample treatment independently. From each of the three QC, different aliquots were derivatized and analyzed along with the rest of samples.

2.6. Data analysis

All MS data files were exported in the mzXML format. Peak picking was carried out using XCMS R-package (The Scripp Research Institute, La Jolla, CA, USA) based on the centWave algorithm using the following settings: maximum tolerated *m/z* deviation in consecutive scans, 15 ppm; chromatographic peak width, 5-25; scan range, 60-2160 scans; noise, 10,000; prefilter step, at least 3 peaks with intensity > 50,000; *m/z* center of the feature, wMean (intensity weighted mean of the feature *m/z* values); signal-to-noise ratio threshold, 50; integration method, peak limits found through descent on the Mexican hat filtered data; no Gaussian fitted to each peak. After peak picking, peak grouping was performed with the following parameters: bandwidth, 30 scans; and width, *m/z* 0.25. Peaks underwent retention time correction using the "symmetric" algorithm to be peak grouped again and "fill peaks" algorithm was employed. After peak picking and grouping, those variables having RSD higher than 30 % in the QC were excluded.

Before multivariate analysis, the data was normalized using the probabilistic quotation normalization (PQN) algorithm, which is based on the determination of a most probable dilution factor by considering the distribution of the quotients of the signals of a certain sample by those of the QC samples [16].

Multivariate statistical analysis was carried out using SIMCA 13.0 (Umetrics, Umeå, Sweden) where, in all cases, common logarithm was applied to reduce the effect of a few particularly intense signals which may influence the statistical analysis and thus, the data interpretation. Also, data was mean centered and then divided by the square root of the standard deviation, i.e. pareto scaling .

Non-parametric Mann-Whitney U univariate analysis and box-plots were performed in R (http://www.R-project.org) . Benjamini-Hochberg false discovery rate (FDR) was carried out to correct for multiple testing.

2.7. Identification of metabolites

Tentative identification of those signals which were significant in the statistical multivariate analysis of the six different comparisons (obtained by PLS-DA models) was carried out by searching their *m/z* values in different databases (METLIN (http://metlin.scripps.edu), HMDB (http://hmdb.ca), and KEGG (http://genome.jp/kegg)), employing an error width of 20 ppm. In addition, only endogenous metabolites were taken into account, i.e., discarding those coming from exogenous sources such as drugs, pesticides, or those of food or plant origin.

Finally, the identity of compounds that were found in databases was confirmed by LC-MS/MS. Standard solution of those compounds which could be acquired commercially were analyzed under same analytical conditions and their retention times and MS/MS fragmentation was compared to the one obtained in a pooled plasma sample.

3. Results and discussion

3.1. Experimental design and analysis of plasma samples

The aim of this work was to obtain a further understanding on the toxic mechanism that underlies to a chronic administration of cocaine and alcohol alone and combined in young-adult rats by an exploratory analysis of the levels of endogenous compounds present in plasma. This study was performed on the *Wistar* rat model, in which four different rat groups were established: 8, 10, 10, and 9 rats were treated with cocaine (group C), alcohol (group A), combination of cocaine and alcohol (group CA), and saline solution (group S, i.e. the control group), respectively. Plasma was chosen as being advantageous due to its accessibility and the fact that it enables the comparison of findings in a way that can be extrapolated to humans.

An important point to take into account during drug-related studies is how such drugs are administered to the animals. According to Gika *et al.* [13], in most of the metabolomic works aimed to study the effects of alcohol, alcohol was administered to

rodents via oral gavage or in the drinking water. Although the oral route better simulates the human alcohol use, gavage use has limitations related to the high number of infusions and it is prone to cause stress in rats. Regarding oral self-administration models (e.g. having the drugs in the drinking water), the average dose ingested is, in most cases, irregular, and require a larger process which implies exceeding the target age of the animals required at the outset of treatment. In addition, sugary substances are typically employed in ethanol oral models, introducing an additional variable [17]. Therefore, intravenous administration was chosen for the intervention as it enables a better control of a delivery of the drugs and gives a direct insight into the pharmacokinetics as drugs are injected directly in the bloodstream. Models of the intravenous self-administration of drugs have been relied upon for decades due to their capacity to explore the abuse liability or motivational effects of psychoactive substances [18]. Using these models as our guideline, a dose of 15 mg per kg of body weight was chosen for cocaine, this being the average amount that a rat is able to self-administer in a session with a duration similar to the one employed in our design previously published -[19]. On the other hand, the chronic administration of 2 g per kg of bodyweight of alcohol has been shown to increase the metabolism of rats compared to lower doses[20]. Hagman and Eriksson [21] have found that the acute administration of the same dose of alcohol leads to a decrease in the concentrations of several amino acids in rat plasma. Meanwhile, a partial recovery of this loss is noted after chronic administration, mediated by a mechanism of tolerance.

In order to include the possible variability occurring during the sample treatment three aliquots from each of the 37 samples underwent sample treatment individually. Thus, a total of 111 plasma samples were analyzed by a methodology recently developed in our laboratory. This methodology is based on the ultrafiltration of plasma samples to eliminate proteins, and then derivatization of polar compounds with FMOC to be then

analyzed on a fused-core reversed-phase stationary phase, a C18 column, under general gradient conditions [14]. **Figure 1** shows the Total Ion Chromatogram of a "non-injection" analysis (**Figure 1A**), what we can consider a FMOC-blank which also contains a standard in a low concentration, not distinguishable in the TIC (**Figure 1B**) and a QC plasma sample (**Figure 1C**), where it can be seen that there are just few minor peaks coming from the labelling agent (those found in **Figure 1B** but not in **Figure 1A**). It is remarkable the suitability of the employed gradient, as it enabled to elute all the compounds in the analysis time established (before 35 min, *see section 2.4*). In addition, the intensity of the compounds which elute with the dead volume of the system is not so high and the presence of peaks at the first minutes of the chromatogram is minimal, what demonstrates the usefulness of the FMOC-derivatization step carried out in this work, which improves the retention of metabolites found in the analyzed sample.

3.2. Metabolic profiling on plasma samples by RPLC-MS

Once all samples and QCs were analyzed, data treatment was performed according to *section 2.6*. Since the amount of data obtained in metabolomics is rather complex, the best statistical tool to extract useful biological information is multivariate statistical analysis. A total number of 1094 signal features were found for the 111 samples analyzed. Unsupervised Principal Component Analysis (PCA) was used to explore trends occurring in the samples. Six samples (four from the A group, one from the C group and another from the CA group) had to be excluded from the data set as they appeared considerably out of the Hotelling's T^2 ellipse for a 0.05 significance level. The next step consisted on signal features filtering. First, features whose RSD % in QC samples was higher than 30 % were discarded, resulting in 890 signal features. Second, features which corresponded to the employed drugs or related metabolites were also discarded as this produces a bias in the models when endogenous metabolism wants to be studied. Only

three signals related to cocaine metabolism were found in the dataset and thus, were eliminated: *m/z* 304.1555 and *m/z* 305.1585 both at 6.8 min coming from cocaine protonated molecular ion and its second most abundant ion from the isotopic profile, respectively, and *m/z* 290.1400 at 6.0 min from benzoylecgonine, one of the main metabolites of cocaine .

The PCA on a data matrix consisting of 887 variables (i.e. signal features) is shown in **Figure 2A**. It shows a slight grouping based on the experimental groups but no tight clustering of the samples. This is a common phenomenon, since the main variance in the data is no necessarily related to a study design [29] [22]. The reason why the QC samples appear together but do not cluster perfectly can be due to the fact that, as explained in *section 2.5*, the QC was not a multiple injection of the same vial along the sequence, thus it shows the real variability including, not only the instrumental variability but also the sample preparation variability. This can be assumed as adequate as the QC is spread on the first component which only covers 24.7 % of the total variability explained by the model. In addition, when plotting the total abundances of all the variables per sample, according to injection order, the resulting plot demonstrates that the method was repeatable along the sequence (see **Figure S1**). Other aspects regarding the reliability of the analytical methodology are included in our previous work [14], in which precision was assayed for a group of 30 known metabolites (amino acids and amines) found in plasma samples by means of instrumental repeatability, method repeatability, and intermediate precision. In all cases RSD values were adequate.

Supervised partial least square discriminant analysis (PLS-DA) model was first built taking into account the four studied groups. As seen in **Figure 2B**, the four groups were grouped separately although there was some overlap, being the main distinction between groups observed in cocaine (C and CA) and non-cocaine-containing groups (A

and S). The quality parameters were $R^2X = 0.401$, $R^2Y = 0.841$, and $Q^2 = 0.598$ and the F and p-values of the cross validated ANOVA (CV-ANOVA) were 9.8 and 3.9×10^{-32}, respectively, thus, there is evidence that there are statistical significant differences in the metabolic profiles of the four studied groups. To demonstrate that the differences found in the PLS-DA model were due to real differences in metabolic profiles and not due to data overfitting, response permutation testing and CV score plot of PLS-DA model were performed. As can be seen in **Figure 2C**, all Q^2 and R^2 values are below the original values (dots on the right side of the plot), demonstrating the validity of the model. Moreover, samples in the CV score plot (**Figure 2D**) are allocated in a similar way than they are in the PLS-DA model (**Figure 2B**), reinforcing the robustness of the proposed model. Another test to validate the model is to predict a third of samples until all samples have been predicted at least once. The percentage of well predicted samples is adequate (99 ± 2 %), highlighting the good power of prediction of this model.

3.3. Selection of influencing variables and metabolite identification

In order to obtain further knowledge on what metabolites are affected by alcohol and/or cocaine administration, first, variables which significantly differ between groups must be highlighted. As the PLS-DA model build on four groups (**Figure 2B**) cannot reveal subtle differences existing among all the experimental groups, different PLS-DA models were built in a pairwise way. Six different PLS-DA models were investigated: drug treatments vs control group (A vs S, C vs S, and CA vs S) and drug treatments among each other (A vs C, A vs CA, and C vs CA). **Figure 3** shows the PLS-DA models with the quality parameters obtained for each pairwise comparison. The highest R^2X and R^2Y values were found in the A vs S model whereas that the lowest values were found in the C vs CA comparison. Overall, Q^2 values were quite high in all models (above 0.812 in all cases except for the C vs CA model). Cross-validated score plots of PLS-DA models are

displayed on **Figures S2**, where it can be seen how the samples are placed in a similar way than they appear in the PLS-DA models (**Figure 3**), reinforcing the robustness of the proposed models. Due to the fact that there was a good separation among the studied groups in the PLS-DA models, it is clear that there are significant differences in the metabolic patterns from the studied groups, thus, a subset of variables can be established for each of the studied comparisons. To this end, the most differentiating variables were selected according to the variable importance in the projection (VIP) value for each of the proposed PLS-DA models. Selecting those variables with VIP values higher than 1.5 resulted in a total of 137 statistically significant variables (after excluding variables coming from signal features corresponding to ions of the isotopic profiles) which corresponded to 107 different compounds, as sometimes the possible compound had more than a statistically significant signal, e.g. fragments, adducts (including FMOC-adducts), etc.

The *m/z* of the protonated molecular ions resulting 107 signals were looked up in different databases (see *section 2.7*). The results of the search yielded a total of 120 different compounds of endogenous origin which corresponded to only 52 molecular formulas (see **Table S1**). The main finding from this table is the fact that most of these metabolites appeared as a FMOC-molecule, meaning that these metabolites would have not been highlighted as significant without FMOC-labelling, as they would not have had retention on the C18 column. As seen in **Table S1**, these metabolites are mainly amino acids and derivatives, dipeptides, and polyamines. Some metabolites, such as dipeptides Asn-Arg (or Arg-Asn) and Ser-Arg (or Arg-Ser) are remarkable for their high VIP values in almost all pairwise comparisons (above 2.0 in most of the cases) (see **Table S1**).

From these 120 possible metabolites 32 standards were acquired to check their retention time and MS/MS spectra which, at best, could be used to identify up to 24

metabolites. However, not all 24 metabolites could be identified, being eight the number of unequivocally identified through confirmation with the analysis of standards (carnosine, spermidine, methionine, cystathionine, argininosuccinic acid, trans-4-hydroxyproline, N-ε-acetyl-L-lysine and serotonin), whereas that three (2-methyl-L-tryptophan, N1-acetyl-spermidine, and β-leucine) could be only tentatively assigned by matching the MS/MS spectra of the $[M+H]^+$ ions to the ones in the MS/MS libraries. Among the different metabolites from **Table S1**, that could not be identified, but were assigned a very tentative identification there were steroids such as estriol-17-glucoronide, bilirubin or its derivatives, serotonin-derived metabolites (6-sulfatoyxmelatonin), and a wide variety of dipeptides. However, since those metabolites could not be unequivocally identified further investigation will not be carried out.

In order to obtain additional information on the behavior of the selected eight metabolites, box-plots (see **Figure S3**) and p-values from the Mann-Whitney U test univariate analysis were also employed in addition to the VIP values obtained from the multivariate PLS-DA. **Table 1** shows the unequivocally identified metabolites, along with their KEGG-ID number, the metabolic pathways where they take place in, the VIP values, the p-values of non-parametric univariate Mann-Whitney U test, and the direction of the regulation for each pairwise comparison whenever it has been significant (either by uni- or multivariate analysis). Usually, high VIP values match low p-values in univariate tests but results might not fully overlap ~~[30]~~ [23]. As seen in **Table 1**, on the one hand, all metabolites with VIP > 1.5 resulted in significant p-values, except for the CA vs S, C vs CA, and A vs C comparisons for carnosine and the A vs S comparison of serotonin that they were below 0.05 but not below the FDR cutoff (0.021). On the other hand, the common trend was that low p-values did not directly translate in VIP values higher than 1.5. This can be justified due to the fact that in multivariate analysis relations

between metabolites and complementary behavior related to a biological problem are taken into account whereas that in the univariate approach independent changes in metabolites are only addressed [23]. For this reason, when investigation of a biological question is required, it is desired to use both, uni- and multivariate analysis, as they may provide complimentary results.

3.4. Biological relevance of the significant metabolites

The final step in the metabolic workflow is to find the biological relevance of the eight metabolites unambiguously identified, which were significantly differentiating the studied groups, as a possible way of investigating the effects of the studied drugs. As seen in **Table 1**, amino acids-related pathways were mainly affected by administration of cocaine and/or alcohol. On the one hand, rats treated with alcohol (A or CA groups) had higher values of carnosine, spermidine, N-ε-acetyl-L-lysine and serotonin than at least a non-containing alcohol group (C and/or S). Methionine is the only of the eight metabolites whose levels decrease in alcohol-containing groups. On the other hand, rats treated with cocaine (C or CA groups) had lower values of argininosuccinic acid, cystathionine and N-ε-acetyl-L-lysine, and higher values of methionine, than at least a non-containing group (A and/or S). Some of the features of these metabolites, such as their role in the different metabolic pathways, will be briefly described.

The dipeptide carnosine and the polyamine spermidine might play a protective role against the damage caused by oxidative stress from alcohol metabolism ~~[31]~~ [24]. Carnosine is mainly present in tissue and it is metabolized by serum carnosinase. Due to the fact that this enzyme is not present in rodents it is possible to find carnosine in blood [25]. Pretreating rats with carnosine before ethanol exposure leads to an acceleration in the recovery from oxidative damage; this is seen both biochemically and histologically [26]. After acute treatment with ethanol (2 g/kg injected intraperitoneally), no changes in

the levels for carnosine were observed in plasma, although and increase of this metabolite was observed in heart tissue [27], this being in accordance to what was observed in plasma (in A vs S) in this work. Regarding the polyamine spermidine, Zaitsu *et al.* observed an increase of this metabolite with respect to the control group in cocaine-treated rats (10 mg/kg intraperitoneal injection) in plasma [12]. The fact that our approach did not observe statistically significant differences either by uni- or multivariate analysis in the C vs S group could be because their dose duration and procedure of administration differ from ours.

Both methionine and cystathionine are metabolites derived from homocysteine. Stabler *et al.* [28] found that cystathionine levels increased in the serum of patients with cobalamin and folate deficiency. Low serum folate levels were observed in a high percentage of alcoholics [29]. This folate deficiency exacerbates the damage to the liver delivered by the alcohol. Hepatic cells metabolize the modified methionine, leading to oxidative damage [30]. This is in agreement to what our results show. Possibly as a consequence of this event, methionine levels in the A group had the opposite behavior.

Argininosuccinic acid is a basic amino acid precursor of arginine in the urea cycle. Cocaine interacts with CYP3A4, cytochrome, enzyme involved in the metabolization of argininosuccinate onto fumarate and arginine, and this could be an indirect way of inhibiting NO synthesis. Acute and binge cocaine treatment increases the production of enzyme inducible nitric oxide synthase in liver, spleen and lung and the nitrite levels in plasma [31], probably, in a metabolic attempt to compensate the reduction of NO levels. This is according to our results, argininosuccinic acid is depleted in the cocaine groups (C and CA) when compared to the control group (S). Aoki *et al.* [32] suggest that liver injury induced by cocaine is mediated by nitric oxide and reactive oxygen species.

It has been reported that the oxidation and degradation of proline and other amino acids is enhanced by oxidative stress [33], which might result in formation of 4-hydroxyproline. Levels of hydroxyproline in urine were also found to be higher in alcohol-treated mice when compared to the control group in a recent metabolomics publication [34].

The reinforcing effects of cocaine, quantified by a conditioned place preference procedure, are attenuated by systemic or intra-accumbens of BRD4 (bromodomain-4) inhibition [35]. The bromodomains are protein domains which recognize acetylated lysine residues. The N-ε-acetyl-lysine present at the ends of histones enables these epigenetic changes.

Serotonin**,** is an important neuroactive molecule present in the CNS, platelets and enterochromaffin cells in the digestive tract. Cocaine binds to serotonin transporters elevating synaptic levels of this neurotransmitter [36] contributing to rewarding effect of this substance. Even though significant differences were not found between the level of this metabolite in the C and S groups, univariate analysis revealed statistical differences between the CA and the S group and multivariate analysis revealed differences between the A and the S group, being in both cases down-regulated in the control group. Patkar *et al.* found, in patients with alcohol dependence, than at the beginning of the withdrawal serotonin plasma levels were similar to the control group but decreased on days 1, 7, and 14 of withdrawal [37].

In summary, the common trend for most of the studied metabolites was that when there were differences between a drug administrated separately (C or A) *vs* the control group the CA group was also different from the control group, behaving in the same way as that drug administrated on its own. However, two metabolites, methionine and N-ε-acetyl-L-lysine, had an interesting trend. On the one hand, while methionine was up- and

down-regulated in the C and A groups, when compared to control group, respectively, the CA group seemed to show a compensated effect of both drugs, but still appeared down-regulated when compared to the control group. On the other hand, N-ε-acetyl-L-lysine was down- and up-regulated in the C and A groups *vs* the control group, respectively, and the CA group behaved as the C group instead of having the same trend as the A group, and did not show a compensated effect as seen in methionine.

4. Concluding remarks

In this work, the first metabolomics study on combined administration of cocaine and alcohol is presented. The use of supervised multivariate analysis (PLS-DA) ~~we~~ dissected some real biological differences between the four studied groups (cocaine, alcohol, cocaine + alcohol, and the control group). A subset of metabolites with VIP values above the cut-off could be extracted from the pairwise PLS-DA models where eight metabolites were unequivocally identified: carnosine, spermidine, methionine, cystathionine, argininosuccinic acid, trans-4-hydroxyproline, N-ε-acetyl-L-lysine, and serotonin. These metabolites are mainly related to the metabolism of different amino acids, such as tryptophan, arginine, proline, methionine, and cysteine metabolism, among others. Our data suggest that processes such as oxidative stress could be affected by a chronic drug intake, in agreement with previously reported literature. Statistical differences in plasmatic levels of most of the identified metabolites were found between cocaine or alcohol administrated individually *vs* combined. This finding can be used to broad the available information of the effects of drug intake. Moreover, these results are the starting point for further investigations needed to better understand the mechanisms of cocaine and/or alcohol administration. Future investigation in our research group will include increasing the number of sampling, evaluating whether there are differences between rat gender, and performing target profiling analysis.

Conflicts of interest

Authors declare that they have no conflict of interest

Acknowledgments

Authors thank financial support from Spanish Ministries of Economy and Competitiveness (project CTQ2013-48740-P) and Health, Social Services and Equality (Carlos III Health Institute, Spanish Network on Addictive Disorders, project RTA-RD12/028/0020, and National Plan on Drug Abuse, project PNSD-2012I057), the University of Alcalá (project CCG2014/EXP-059), and the UNED Plan for Research Promotion. Elena Sánchez-López thanks the University of Alcalá for her pre-doctoral contract.

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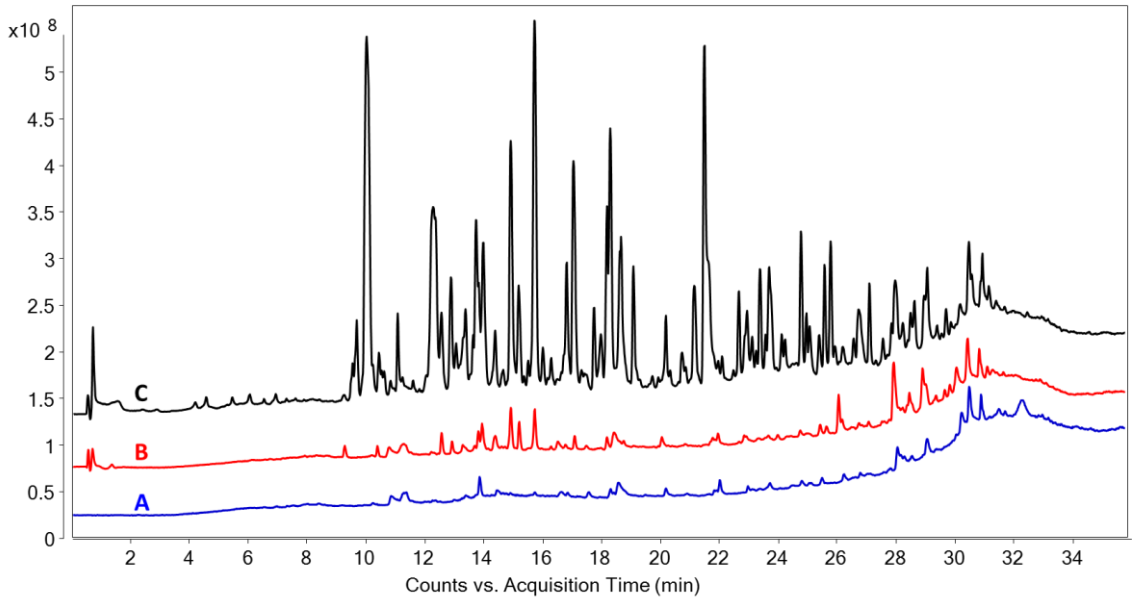
Figure captions.

Figure 1. Total Ion Chromatogram of a "non-injection" analysis (A), what can be considered a FMOC-blank which also contains a standard in a low concentration (not distinguishable in the TIC) (B) and a QC plasma sample (C).

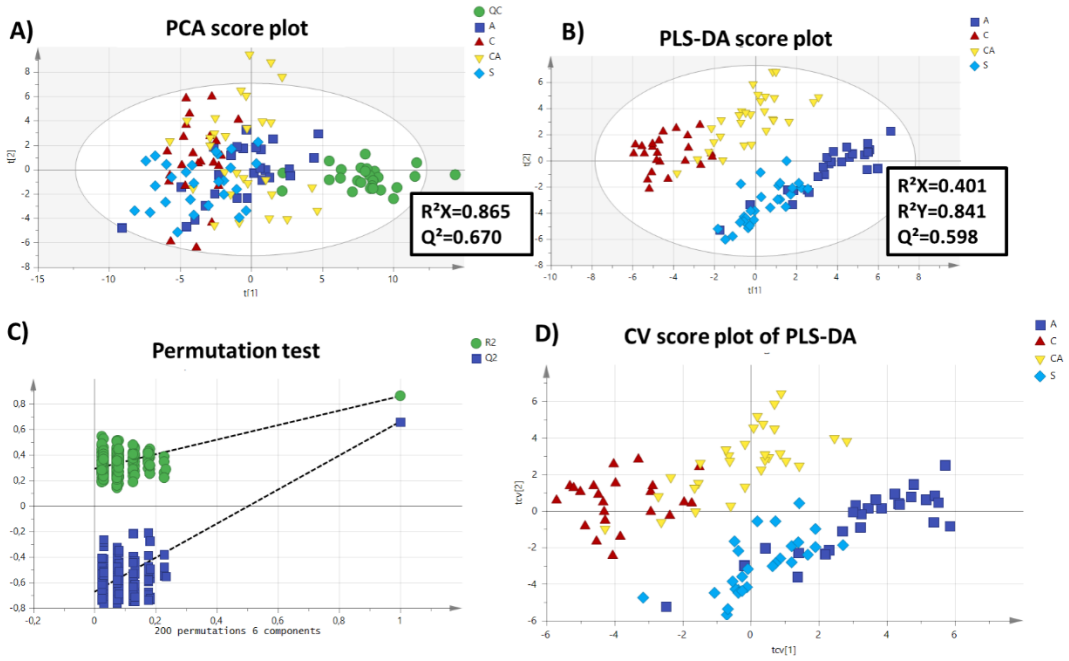
Figure 2. (A) PCA of the four studied groups of samples and the QC samples; (B) PLS-DA of the four studied groups; (C) permutation test performed on the PLS-DA model (200 permutations); and (D) CV score plot of the PLS-DA model. C: cocaine group, A: alcohol group, CA: cocaine and alcohol group, and S: saline group (control group).

Figure 3. PLS-DA models for the six different pairwise comparisons of the studied groups (C vs S, A vs S, CA vs S, C vs CA, A vs C, and A vs CA), where C: cocaine group, A: alcohol group, CA: cocaine and alcohol group, and S: saline group (control group).

Figr-1**Figure 1.**



Figr-2**Figure 2.**



Figr-3**Figure 3.**

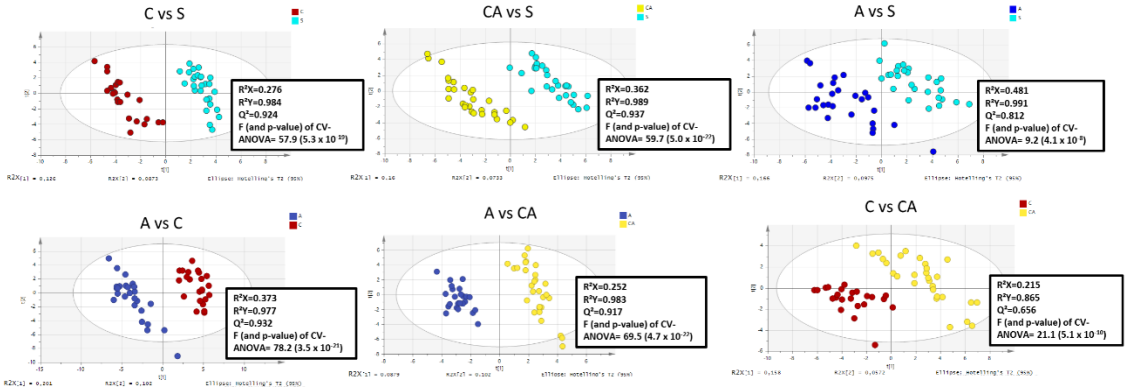


Table 1. Metabolites which were unequivocally identified in the metabolomics study, along with their KEGG-ID, the related metabolic pathways and the direction of the regulation according to the VIP values and the p-values from non-parametric univariate Mann-Whitney U analysis for each of the six pairwise comparisons.

Metabolite	**KEGG ID**	**Related metabolic pathways**	**Direction of the regulation according to the VIP values, and p-values from non-parametric univariate Mann-Whitney U to the pairwise comparison***					
			C vs S	**A vs S**	**CA vs S**	**C vs CA**	**A vs C**	**A vs CA**
Carnosine	C00386	Histidine metabolism. β-Alanine metabolism.	- VIP: 0.17 p-value: $5.8 \cdot 10^{-1}$	**↑A** VIP: **2.29** p-value: **$1.8 \cdot 10^{-2}$**	**↑CA** VIP: **1.50** p-value: $4.3 \cdot 10^{-2}$	**↓C** VIP: **1.80** p-value: $2.7 \cdot 10^{-2}$	**↑A** VIP: **1.69** p-value: $2.7 \cdot 10^{-2}$	- VIP: 0.62 p-value: $4.0 \cdot 10^{-1}$
Spermidine	C00315	Arginine and proline metabolism	- VIP: 0.80 p-value: $8.0 \cdot 10^{-2}$	**↑A** VIP: **2.38** p-value: **$2.7 \cdot 10^{-6}$**	**↑CA** VIP: 1.32 p-value:**$1.5 \cdot 10^{-4}$**	**↓C** VIP: 0.74 p-value: **$1.3 \cdot 10^{-2}$**	**↑A** VIP: 1.13 p-value: **$2.6 \cdot 10^{-3}$**	- VIP: 0.68 p-value: $7.1 \cdot 10^{-1}$
Methionine	C00073	Cysteine and methionine metabolism. Glucosinolate biosynthesis. Aminoacyl-tRNA biosynthesis. 2-Oxocarboxylic acid metabolism. Protein digestion and absorption. Mineral absorption. Central carbon metabolism in cancer.	**↑C** VIP: 0.71 p-value: **$1.9 \cdot 10^{-2}$**	**↓A** VIP: 1.39 p-value: **$6.7 \cdot 10^{-5}$**	**↓CA** VIP: 0.83 p-value: **$2.5 \cdot 10^{-3}$**	**↑C** VIP: **1.51** p-value: **$1.8 \cdot 10^{-6}$**	**↓A** VIP: 1.33 p-value: **$3.1 \cdot 10^{-7}$**	- VIP: 0.59 p-value: $8.1 \cdot 10^{-2}$
Cystathionine	C02291	Glycine, serine, and threonine metabolism. Cysteine and methionine metabolism.	- VIP: 0.91 p-value: $6.4 \cdot 10^{-2}$	- VIP: 1.05 p-value: $5.8 \cdot 10^{-2}$	- VIP: 0.66 p-value: $2.1 \cdot 10^{-1}$	- VIP: 0.23 p-value: $6.9 \cdot 10^{-1}$	**↑A** VIP: 1.37 p-value: **$2.2 \cdot 10^{-3}$**	**↑A** VIP: **1.78** p-value: **$1.4 \cdot 10^{-3}$**
Argininosuccinic acid	C03406	Arginine biosynthesis (urea cycle). Alanine, aspartate and glutamate metabolism.	**↓C** VIP: 1.12 p-value: **$1.2 \cdot 10^{-2}$**	- VIP: 0.25 p-value: $9.4 \cdot 10^{-1}$	**↓CA** VIP: **1.67** p-value: **$5.8 \cdot 10^{-5}$**	- VIP: 0.78 p-value: $8.0 \cdot 10^{-2}$	**↑A** VIP: 1.01 p-value: **$4.5 \cdot 10^{-3}$**	**↑A** VIP: **2.16** p-value: **$3.2 \cdot 10^{-5}$**
Trans-4-hydroxyproline	C01157	Arginine and proline metabolism	- VIP: 0.67 p-value: $2.3 \cdot 10^{-1}$	- VIP: 1.15 p-value: $9.6 \cdot 10^{-2}$	- VIP: 0.65 p-value: $5.8 \cdot 10^{-1}$	- VIP: 0.11 p-value: $3.7 \cdot 10^{-1}$	**↑A** VIP: 1.32 p-value: **$4.2 \cdot 10^{-3}$**	**↑A** VIP: **1.76** p-value: **$2.1 \cdot 10^{-2}$**
N-ε-acetyl-L-lysine	C02727	Lysine degradation	**↓C** VIP: 1.06 p-value: **$6.2 \cdot 10^{-4}$**	**↑A** VIP: 1.28 p-value: **$6.6 \cdot 10^{-4}$**	**↓CA** VIP: 0.73 p-value: **$9.3 \cdot 10^{-3}$**	- VIP: 0.34 p-value: $2.5 \cdot 10^{-1}$	**↑A** VIP: **1.58** p-value: **$1.8 \cdot 10^{-8}$**	**↑A** VIP: **2.04** p-value: **$6.5 \cdot 10^{-8}$**
Serotonin	C00780	Tryptophan metabolism. Biosynthesis of alkaloids derived from shikimate pathway. cAMP signaling pathway. Neuroactive ligand-receptor interaction. Gap junction.	- VIP: 1.24	**↑A** VIP: **1.67**	**↑CA** VIP: 0.64	- VIP: 0.34	- VIP: 0.21	- VIP: 0.62

		Synaptic vesicle cycle. Serotonergic synapse. Taste transduction. Inflammatory mediator regulation of TRP channels. Bile secretion. Serotonin receptor agonists/antagonists. Melatonin biosynthesis.	p-value: $1.1 \cdot 10^{-1}$	p-value: $4.1 \cdot 10^{-2}$	p-value: $\mathbf{8.0 \cdot 10^{-3}}$	p-value: $2.6 \cdot 10^{-1}$	p-value: $8.8 \cdot 10^{-1}$	p-value: $7.3 \cdot 10^{-1}$

*Regulation is only displayed for those whose VIP > 1.5 or p-value of Mann-Whitney U test < FDR cut-off (0.021).