Prescription and illicit psychoactive drugs in oral fluid—LC–MS/MS method development and analysis of samples from Brazilian drivers

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A B S T R A C T
This study is part of a larger project designed to investigate the prevalence of psychoactive drug (PAD) use among Brazilian drivers. In this paper we describe the development and validation of an analytical method to analyze 32 prescription and illicit PADs (amphetamine, benzodiazepines, cocaine, cannabis, opioids, ketamine and m-CPH) and metabolites in oral fluid samples collected with a QuantisalTM device. Samples were extracted with ethyl acetate:hexane and analyzed by LC–MS/MS. Instrument LOD ranged from 0.26 to 0.65 ng/mL. Mean procedural recoveries at 1.3 ng/mL (LOQ) ranged from 50% to 120% for 24 compounds. Recoveries were concentration independent, with the exception of fenproporex, heroin and ecorpine methyl-ester (EME) for which the recovery decreased significantly at higher levels (13 and 52 ng/mL). Ion suppression decreased on the matrix was <20% for most compounds, and higher than 60% for EME and diethylpropion. Analysis was performed against a in-matrix standard curve. About 10% of the 2235 oral fluid samples collected from drivers on Brazilian Federal highways were positive (>LOD) for at least one analyte investigated. Alone or in combination with other drugs, cocaine/metabolites were the analytes most detected in the samples (129; 5.8%), followed by amphetamines/metabolite (69; 3.1%), benzodiazepines (28; 1.2%), cannabinoids (23; 1.1%) and opioids (8; 0.4%). Detection of at least two PADs from different classes accounted for 9.3% of the 236 positive samples. Cocaine was found at higher levels in the samples (up to 1165 ng/mL). Preventive measures aimed at reducing the use of PADs by drivers in Brazil will certainly contribute to decrease the country’s highway death rates.

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1. Introduction

In the last decade, there has been a significant worldwide increase in the use of illicit drugs and the abuse of prescription drugs, such as benzodiazepines, barbiturates, and amphetamines [1]. The increased risk of driving under the influence (DUI) of these psychoactive drugs (PADs) has been substantially demonstrated in the literature [2–7]. In Brazil, 11% of the truck drivers interviewed reported using amphetamines in the state of Mato Grosso do Sul [8] and 66% in the state of Minas Gerais [9]. Biological samples from truck drivers have tested positive for various substances, including cannabinoids, cocaine and amphetamines in São Paulo state [10–12]. Traffic accidents were the second highest cause of death in the country in the last three decades, representing 20.3 deaths per 100 000 inhabitants in 2007 [13].

In the last 10 years, the use of oral fluid to monitor the use of PAD by humans has been proved to have a number of advantages over the traditional urine and blood matrices. Oral fluid sampling is fast, easy and less intrusive for drivers [14]. It allows the detection of the drug in a non-metabolized form and, compared to serum analysis, is potentially more sensitive to basic drugs and drugs with low plasma protein binding, such as amphetamines and cocaine [3,15–18]. Some disadvantages regarding the use of oral fluid include the reduced sample volume, high content of glycogen (oral mucin), and low concentration of drugs strongly bound to plasma proteins, such as benzodiazepines and ∆2-THC (tetrahydrocannabinol).

The commercial oral fluid collection system contains buffers with stabilizing salts, non-ionic surfactants for surface wetting, and antibacterial agents which guarantee good stability for most drugs and their metabolites during storage at 4 °C [19]. However, these devices excessively dilute the samples, and thus a sensitive

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Table 1
Optimized conditions for LC–MS/MS, precursor and product ions, ion ratio and respective RSD (%), and retention time for each psychoactive drug and metabolite analyzed.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Precursor ion (m/z)</th>
<th>Product ion (m/z)*</th>
<th>DP (V)</th>
<th>CE (V)</th>
<th>EP (V)</th>
<th>CPX (V)</th>
<th>IR average (RSD, %), n = 24</th>
<th>RT (min)</th>
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<td></td>
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<tr>
<td>Zolazepam (IS)</td>
<td>287</td>
<td>243</td>
<td>86</td>
<td>51</td>
<td>11</td>
<td>20</td>
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<td>281</td>
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<td>121</td>
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<td>121</td>
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<td>51</td>
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<tr>
<td>m-CPP</td>
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<td>8.9</td>
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</table>

* Quantifier ion underlined; V, volts; IS, internal standard; DP, declustering potential (DP); CE, collision energy; EP, entrance potential; CPX, collision cell exit potential; IR, ratio between the quantifier and qualifier ions; RSD, relative standard deviation; RT, retention time; MBDB, n-methyl-1-(1,3-benzodioxol-5-il)-2-butanamine; MDA, 3,4-methylenedioxymetamphetamin; MDEA, methylene dioxyxylmorphatin; MDMA, 3,4-methylenedioxymethamphetamine; and m-CPP, 1-(3-chlorophenol) pipеразин.

detection method, such as LC–MS/MS, needs to be used for quantification. Sample extraction methods include liquid–liquid extraction (LLE) [20–24], and solid phase extraction (SPE) [19,25,26].

The aims of this study were to develop an analytical method for PADS from various chemical classes by LC–MS/MS in oral fluid, and to apply this method to analyze samples collected from Brazilian drivers in the first nationwide survey conducted in the country.

2. Materials and methods

2.1. Psychoactive drug standards and reagents

Standards of dl-diethylpropion (DIE) hydrochloride (HCI) and dl-femproporex HCl (FEM) were kindly donated by Achê Pharmaceutical Laboratories S.A. (São Paulo, Brazil), and dl-threo-methylphenidate HCl (MPH) by Novartis Pharma (São Paulo, Brazil). Alprazolam, bromazepam, clonazepam, diazepam, flunitrazepam, lorazepam, midazolam, nizgasepam, oxazepam and temazepam were purchased from Roche Pharmacuetical (Anápolis, Brazil); codeine phosphate, methadone HCl, morphine sulfate and tramadol HCl from Cristália Pharmaceutical (Itapira, Brazil); atropine sulfate monohydrate from Henrifarma (São Paulo, Brazil); ethyl morphine HCl from Merck (Darmstadt, Germany); zolazepam HCl from United States Pharmacoeuna, and α-amphetamine HCl and Δ1-THC from Lipomed (Arlesheim, Switzerland). Standard solutions of 1 mg/mL of dl-methamphetamine (METH), dl-3,4-methylenedioxymetamphetamine (MDA), dl-3,4-methylenedioxymethamphetamine (MDMA), n-methyl-1-(1,3-benzodioxol-5-il)-2-butanamine (MBDB), dl-3,4-methylenedioxymethamphetamine (MDMA), ketamine, heroin, canabinol, cocaine, benzylophone (BZE), ergonime methyl-ester (EME), nizgasepam and 1-(3-chlorophenol)piperazin were provided by the Brazilian National Institute of Criminalistics (Brasília, Brazil).

PAD and metabolite stock solutions at 1 mg/mL were prepared in ethyl acetate (benzodiazepines), acetonitrile (opioids) or methanol. Working solutions were prepared by diluting the stock solution in methanol at final concentrations of 3.75 µg/mL and 25 ng/mL. Stock and working solutions were stored at −15 ± 4 °C. In-matrix standard solutions at concentrations ranging from 0.5 to 20 ng/mL were prepared by spiking a blank oral fluid extract.

Methanol (MeOH) and acetonitrile HPLC grade and ultrapure ammonium formate (98% purity) for HPLC were obtained from Sigma–Aldrich (St. Louis, USA), ammonium carbonate and hexane from Mallincrodt (Phillipsburg, USA), ethyl acetate HPLC grade and ammonium hydroxide concentrate p.a. from Merck (Darmstadt, Germany). High purity water was obtained from a Milli-Q water system (Billerica, USA). Quantitative oral fluid collection devices, filters and preservative buffer solution were purchased from Immunalysis Corporation (Pomona, CA, USA). Each device contained a collection pad with an indicator that turned blue when 1 mL of oral fluid was collected, and a plastic transport tube with 3 mL of preservative buffer (final specimen volume of 4 mL).
2.2 Sample preparation and extraction

The cotton pad containing the oral fluid was squeezed and the released solution poured into a glass container to freeze. The LLE sample extraction was based on the method described by Ørstad et al. [21]. In summary, a 0.50 mL aliquot of the sample was transferred to a 2 mL Eppendorf tube, 50 μL of saturated ammonium carbonate solution (pH 9.3) added, the tube vortexed to mix, 1.3 mL of ethyl acetate:hexane (4:3) added and the tube vortexed for 20 s and stirred in a shaker for 10 min. A 1.0 mL aliquot of the organic phase was concentrated to dryness under nitrogen at 50 °C. The residue was resuspended for analysis in LC–MS/MS in 250 μL of initial mobile phase with internal standards (ISs) [zolazepam, atropine and ethyl morphine at 25 ng/mL]. Oral fluid samples were thawed and immediately extracted. All samples above the in-matrix standard curve concentration range were diluted and reanalyzed.

2.3 LC–MS/MS analysis

Analyses were performed in a Shimadzu LC-20AD (Kyoto, Japan) liquid chromatographer coupled with a mass spectrometer Applied Biosystems/MDS ScieX 4000 QTRAP MS system (Foster City, USA), with an electrospray interface (ESI). The chromatographic separation was performed at 40 °C with a Luna® C18(2) column, 150 mm × 2.0 mm, 5 μm (Phenomenex; Torrance, USA). The injection sample volume was 10 μL. The mobile phase, delivered at a flow rate of 0.2 mL/min, was a gradient of water with 5 mmol/L ammonium formate (solvent A), and MeOH with 20% of acetonitrile and 5 mmol/L ammonium formate (solvent B), programmed as follows: 32.5% (solvent B) during 2 min linearly increased to 75% in 6.5 min, 80% in 7.4 min, 95% in 13–13.2 min, 100% in 17 min, decreased to original conditions for 5 min, which resulted in a total chromatographic run time of 22 min.

The MS/MS ion source was operated in positive ESI mode at 600.0 °C with the nebulizer and heater gas set to 45.0 psi. Ion spray voltage was set to 4,500 V, curtain gas at 100 psi, and collision gas to HIGH. Positive ionization (ESI+) was performed in the Schedule MRM (multiple reaction monitoring) mode, range of detection for 120 s, obtaining the transition from two fragments for each analyte (quantifier and qualifier ions) [27]. The overall cycle time for 67 MRM transitions (including three for internal standards) was 2.5 s and 528 cycles/scan. The MRM transitions, collision energies and other target-dependent parameters for each target were optimized by direct infusion of the corresponding standard solutions at concentrations of 50–200 ng/mL in the mobile phase (MeOH:H2O) and ammonium formate 5 mmol/L, according to manufacturer instructions. The resolutions for the selection of the precursor ions in Q1 and the product ions in Q3 were set to UNIT mass. Data processing was performed using the Analyst Version 1.5.1 software.

2.4 Method validation

Linearity of the response and selectivity (interfering signals) of the method were evaluated by analyzing a blank oral fluid extract [pool from 10 volunteers] [28]. Linearity was evaluated using in-matrix standard solutions at levels of 0.5, 1.0, 1.5, 2.5, 5, 10, 15 and 20 ng/mL (n = 3 at each level) by plotting the peak area ratio of an analyte/IS using weighted (1/x2) linear least-square regressions. The 5% of intercept was calculated as linear coefficient × 100/(angular coefficient × yintercept) and the regression precision as standard deviation of residues × 100/yintercept. Within the linearity study, the ratios between the quantifier and qualifier ions were calculated for each compound at each level (n = 24).

Fig. 1. LC–MS/MS ions chromatograms of a blank oral fluid sample extract spiked at concentration of 0.5 ng/mL MBDB, n-methyl-1-(1,3-benzodioxol-5-il)-2-butanamine; mCPP, 1-(3-chlorophenyl)piperazine; MDA, 3,4-methylenedioxy amphetamine; MDEA, methyline dioxyethylamphetamines; MDMA, 3,4-methylenedioxymethamphetamine; THC, tetrahydrocannabinol; BZE, benzoylcegonine; and EME, ecgonine methyl-ester.
The limit of detection (LOD) of the instrument was defined as the lowest concentration of each analyte spiked in a blank oral fluid extract (n = 5), which gave a response of a quantitative ion equal to 3 times the signal-to-noise ratio (S/N), calculated by the instrument software. The limit of quantification (LOQ) of the instrument was defined as before, but considering 10× S/N. In both cases, the qualitative ion should give a response of at least 2× S/N. Extraction recovery was determined by comparing the response of the fortified extracted samples (prepared by spiking with the standard solution a blank oral fluid sample before extraction) with the response in samples fortified after extraction, not corrected internal standard [29]. The extraction recovery was determined at three levels for each compound (n = 6 at each level) and used to assess accuracy of the method (bias). The relative standard deviation (RSD) of the data was used to assess intra-day precision (repeatability). The method was satisfactorily validated and considered to be quantitative for compounds for which the mean extraction recovery was within the range of 50–120%, and RSD < 20% [30,31]. LOQ of the method for each compound was defined as the lowest level for which the method was validated.

Matrix effects were evaluated by two different ways [28]: (a) comparing the analyte signal in the solvent with the analyte signal in the oral fluid matrix (pool from 6 volunteers) fortified after extraction, and the result expressed as a percentage of ion suppression or enhancement in comparison to a solvent solution; (b) visual observation of the detector response of a continuous post-column infusion (10 µL/min) of a solvent and in-matrix solution of THC (10 ng/mL), cocaine and diazepam (5 ng/mL).

The stability of the processed samples containing 16 ng/mL analyte concentration arranged in the chromatograph rack at 10 °C was investigated (n = 3). Samples were analyzed at 0, 24, 36, 56 and 68 h after being processed.

2.5. Oral fluid samples

This study is part of a larger project designed to evaluate the use of PAD among drivers on federal highways in Brazil. In this project, oral fluid samples were collected by trained personnel from 3397 drivers on federal highways in all 26 Brazilian states and the Federal District. Samples were collected with the support of the Brazilian Federal Highway Police on Fridays and Saturdays between 12 pm and 12 am, from August 2008 to September 2009. The project was approved by the Ethics Committee of the Clinical Hospital of Porto Alegre. In the present study, 2235 samples of the overall collected samples were analyzed.

Oral fluid (1 mL) samples were collected using a Quantasorb™ device that uses a pad with a cotton swab which is placed between the subject’s cheek and gum. The cotton pad is transferred to a vial containing buffering solution, then capped, labeled, and transferred to the laboratory in containers with temperature monitored at approximately 5 °C no more than 2 days after sample collection.

### Table 2

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Instrumental, ng/mL LOD</th>
<th>Extraction recovery (RSD), % at 1.3 ng/mL</th>
<th>Extraction recovery (RSD), % at 13 ng/mL</th>
<th>Extraction recovery (RSD), % at 52 ng/mL</th>
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<td>84.0 (6.7)</td>
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<td></td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ketamine</td>
<td>0.26</td>
<td>0.52</td>
<td>56.8 (3.9)</td>
<td>63.5 (3.7)</td>
</tr>
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</tr>
<tr>
<td><strong>Semi-quantitative analysis</strong></td>
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</tr>
<tr>
<td><strong>Amphetamines</strong></td>
<td></td>
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</tr>
<tr>
<td>Amphetamine</td>
<td>0.26</td>
<td>0.65</td>
<td>40.4 (9.8)</td>
<td>44.0 (10.6)</td>
</tr>
<tr>
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<tr>
<td><strong>Opioids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Codeine</td>
<td>0.26</td>
<td>0.65</td>
<td>58.9 (5.8)</td>
<td>94.3 (6.6)</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ketamine</td>
<td>0.26</td>
<td>0.52</td>
<td>39.0 (7.9)</td>
<td>40.4 (15.3)</td>
</tr>
<tr>
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<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cannabinoids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>THC</td>
<td>0.52</td>
<td>1.04</td>
<td>53.9 (3.6)</td>
<td>62.1 (2.9)</td>
</tr>
</tbody>
</table>

MBBD, n-methyl-1-[(3-benzozxol-5-il)-2-butanimine; MDA, 3,4-methylenedioxyamphetamine; MDEA, methylenedioxyethyl amphetamine; MDMA, 3,4-methylenedioxy-methamphetamine; and m-CPP, 1-(3-chlorophenyl) piperazine.
Blank oral fluid samples were obtained using the same procedure for non-drug user volunteers. The samples were not further weighed before analysis.

3. Results and discussion

3.1. Method validation

Table 1 shows the precursor and product ions, fragmentation and optimized ionization conditions, ion ratios and chromatographic retention times of the 32 PADS and metabolites analyzed in this study. The ratios between the quantifier and qualifier ions (IR) were generally stable among the runs and at the different concentration levels, with RSD lower than 15% in most cases (n = 24), and morphone showing the greatest variation (19.1%). The method was shown to be selective under the chromatographic conditions, with no significant interferences from endogenous components at the retention times of the analyzed compounds.

For all compounds, linearity using 1/x² weighted linear regression was satisfactory from 0.5 to 20 ng/ml ($R^2 = 0.9942–0.9995$), with % of intercept ranging from −0.1 to 6.7, and regression precision ranging from 1.8% to 5.9%. The ion chromatograms are shown in Fig. 1. Low signal intensities were obtained for THC and cannabinoil. The chromatographic run lasted 22 min (methanol/acetonitrile mobile phase, 0.3 mL/min), longer than that used by Øiestad et al. [21] also for 32 compounds at the same flow rate using acetonitrile as mobile phase (< 10 min), but shorter than the method used by Wylie et al. [17] for 22 compounds (29 min, acetonitrile).

Table 2 shows the instrumental and method parameters evaluated in the study. Instrumental LOD was 0.26 or 0.52 ng/mL and instrumental LOQ ranged from 0.52 to 1.3 ng/mL. The method was considered to be quantitative for 24 compounds (extraction recovery ≥ 50%), including all 11 benzodiazepines, with a LOQ of 1.3 ng/mL for all compounds except for THC (13 ng/mL). For the other 8 compounds, the extraction recoveries were sufficient only for a semi-quantitative analysis. In general,
recoveries were independent of concentration, with the exception of femproporex, heroin and ecgonine methyl-ester (EME) for which recovery decreased significantly at higher levels. RSD was <20% for all compounds at all spiking levels. Very low extraction recoveries were found for diethylpropion (<6%) and benzoylecgonine (<1%). Øedstad at al. [21] also found very low recovery for benzoylecgonine (0.2%) using ethylacetate:heptane (4:1) in the LLE at similar concentration levels. Wylie et al. [17] found a 62% recovery for benzoylecgonine in oral fluid samples at 200 ng/mL after SPE and LC–MS/MS detection; recoveries at lower levels were not reported.

Fig. 2 shows the matrix effects of each analyte in the oral fluid extract spiked at the 5 ng/mL level. With exception of flunitrazepam and clonazepam, there was a depletion of the response in all cases due to the matrix, which reached 20% for most compounds. Ion suppression was greater for EME and diethylpropion (>60%, in average). RSD (n = 6) of the analyte response in-matrix was <12% in all cases, except for EME (28%). Ion suppression was 38.6% for THC, an effect that is illustrated in Fig. 3. Suppression or enhancement of the analyte response in LC–MS/MS is normally due to the presence of stabilizers and preservatives present in the sample collection device, and to proteins present in the matrix [19]. Although fewer matrix effects are expected in clean samples, Dam et al. [32] observed no signal suppression for morphine in oral fluid collected with a Salivette® after a simple dilution step, and 10–15% ion suppression after SPE extraction; protein precipitation resulted in suppression of 50–70% in some areas of the chromatogram. After direct injection of diluted spiked oral fluid samples, Wood et al. [19] found the most dramatic signal suppression effect for morphine and 6-AM (68–87%), as well as codeine, amphetamine and MDA (33–67%). Concheiro et al. [26] obtained a large matrix suppression effect for amphetamines (46.2–70.7%) even after SPE clean-up. In our study, we used an in-matrix standard curve for the quantification of all investigated analytes to compensate for matrix effects. The stability study has shown that processed samples can be stored at 10 °C for up to 36 h before being analyzed (at least 80% remaining, Fig. 4).

To the best of our knowledge, this is the first multiclass LC–MS/MS analytical method developed for PADS in oral fluid that includes methylphenidate, diethylpropion, femproporex and m-CPP. Souza et al. [33] developed a GC/MS method for the analysis of five amphetamines in oral fluid that include methylphenidate, femproporex and diethylpropion. The commercial immunoassays usually applied on roadside surveys with oral fluid are not able to detect these three compounds, even at high concentrations [33,34].

3.2. Samples analyzed

Oral fluid samples obtained from 2235 drivers on federal highways in 24 of the 26 Brazilian states and the Federal District were analyzed. About 95% of the drivers were men, and were 18–80 years of age (mean age: 37.1 years ± 11.2). About half of them were driving automobiles (50.5%), 29.6% motorcycles, 10.8% busses and 9.1% trucks.

About 10% of the samples (236 samples) were positive (>LOD) for at least one of the 32 analytes investigated, alone or in combination with a metabolite or other drugs. Eleven analytes were not detected (<LOD) in any of the samples analyzed (MBDB, MDA, MDEA, flunitrazepam, midazolam, oxazepam, temazepam, heroin, methadone, ketamine and m-CPP). Table 3 summarizes the results of the 236 positive samples. Concentration was given only to those compounds, which were quantitatively analyzed (Table 2).

Amphetamines and metabolites were found in 69 samples (9 samples with drugs of other classes), corresponding to 3.1% of all samples analyzed (29.2% of positive samples). Amphetamines used as appetite suppressants such as femproporex and diethylpropion are subject to abuse and illicit traffic, being under international control since 1971 [35]. In Brazil, femproporex and diethylpropion were prohibited in 2011 [36], and currently only methylphenidate and lisdexamfetamine are legally prescribed in the country, the latter registered in 2011. Most of the amphetamine detected in the samples is likely the metabolite of other amphetamine drugs analyzed (femproporex, MDMA and METH [37]). Amphetamine alone was detected in 10 oral fluid samples (~2–190 ng/mL), femproporex alone in one sample (~4 ng/mL), and femproporex plus amphetamine detected in 24 samples, of which 1 also had METH, 2 methylphenidate and 3 diethylpropion (Table 3). A semi-quantitative analysis showed that in 18 of these 24 samples, amphetamine levels were higher (~6–1500 ng/mL) than those for femproporex (~2–800 ng/mL) (AMP/FEM ~ 1.2–21). Assuming that all amphetamine detected in these samples came from the use of femproporex, and according to the pharmacokinetics study conducted by Comiran et al. [38], it is most likely that individuals had taken femproporex more than 4 h before the oral fluid samples were collected. Peak femproporex concentrations in oral fluid occur between 1 and 1.5 h after administration (70.7–227.5 ng/mL) and that of amphetamine between 1.5 and 4 h (33.0–150.9 ng/mL) [38].

Benzodiazepines were detected in 28 samples (8 samples with drugs of other classes), corresponding to 1.3% of all samples and
Table 3
Prescription and illicit psychoactive drugs detected (≥LOD) in oral fluid samples collected from Brazilian drivers (N=2235 samples).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Samples detected</th>
<th>% positive samples</th>
<th>% total samples</th>
<th>Range, ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzo diazepines and metabolites</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Only alprazolam</td>
<td>5</td>
<td>2.1</td>
<td>0.2</td>
<td>1.3–3.5</td>
</tr>
<tr>
<td>Only clonazepam</td>
<td>2</td>
<td>0.8</td>
<td>0.1</td>
<td>1.4–1.6</td>
</tr>
<tr>
<td>Only diazepam</td>
<td>3</td>
<td>1.3</td>
<td>0.1</td>
<td>2.2</td>
</tr>
<tr>
<td>Only lorazepam</td>
<td>1</td>
<td>0.4</td>
<td>0.0</td>
<td>2.5</td>
</tr>
<tr>
<td>Only nitrazepam</td>
<td>1</td>
<td>0.4</td>
<td>0.0</td>
<td>8.9</td>
</tr>
<tr>
<td>Only nordiazepam</td>
<td>2</td>
<td>0.8</td>
<td>0.1</td>
<td>b</td>
</tr>
<tr>
<td>Diazepam/nordiazepam</td>
<td>2</td>
<td>0.8</td>
<td>0.1</td>
<td>b</td>
</tr>
<tr>
<td>Diazepam/lorazepam</td>
<td>2</td>
<td>0.8</td>
<td>0.1</td>
<td>b</td>
</tr>
<tr>
<td>Diazepam/MPH</td>
<td>1</td>
<td>0.4</td>
<td>0.0</td>
<td>1.5/2.9</td>
</tr>
<tr>
<td>Diazepam/bromazepam</td>
<td>1</td>
<td>0.4</td>
<td>0.0</td>
<td>b/2.1/31.7</td>
</tr>
<tr>
<td>Cocaine and metabolites</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Only cocaine</td>
<td>48</td>
<td>20.3</td>
<td>2.1</td>
<td>1.0–1165</td>
</tr>
<tr>
<td>Only EME</td>
<td>4</td>
<td>1.7</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>RZE/EME</td>
<td>1</td>
<td>0.4</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Cocaine/EME</td>
<td>5</td>
<td>2.1</td>
<td>0.2</td>
<td>b/9.2–/</td>
</tr>
<tr>
<td>Cocaine/BZE</td>
<td>28</td>
<td>11.9</td>
<td>1.3</td>
<td>b/9.6–/</td>
</tr>
<tr>
<td>Cocaine/BZE/EME</td>
<td>26</td>
<td>11.0</td>
<td>1.2</td>
<td>b/915–/</td>
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<tr>
<td>Cannabinoids</td>
<td>18</td>
<td>7.6</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Only THC</td>
<td>9</td>
<td>3.8</td>
<td>0.4</td>
<td>b/65.5</td>
</tr>
<tr>
<td>Only cannabionol</td>
<td>5</td>
<td>2.1</td>
<td>0.2</td>
<td>b/7.8</td>
</tr>
<tr>
<td>THC/cannabionol</td>
<td>4</td>
<td>1.7</td>
<td>0.2</td>
<td>b/5.6–/1.3</td>
</tr>
<tr>
<td>Opioids</td>
<td>4</td>
<td>1.7</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Only codeine</td>
<td>2</td>
<td>0.8</td>
<td>0.1</td>
<td>b/3.3</td>
</tr>
<tr>
<td>Only morphine</td>
<td>1</td>
<td>0.4</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Only tramadol</td>
<td>1</td>
<td>0.4</td>
<td>0.0</td>
<td>2.6</td>
</tr>
<tr>
<td>Drug combination from different classes</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Cocaine/THC/cannabionol</td>
<td>2</td>
<td>0.8</td>
<td>0.1</td>
<td>b/49.4–/20.2/7.8–14.5</td>
</tr>
<tr>
<td>Cocaine/cannabionol</td>
<td>2</td>
<td>0.8</td>
<td>0.1</td>
<td>b/5.6–/</td>
</tr>
<tr>
<td>Cocaine/clonazepam</td>
<td>2</td>
<td>0.8</td>
<td>0.1</td>
<td>1.45–6.2–/1.3</td>
</tr>
<tr>
<td>Cocaine/AMP</td>
<td>1</td>
<td>0.4</td>
<td>0.0</td>
<td>2.4/</td>
</tr>
<tr>
<td>Cannabinol/EME</td>
<td>1</td>
<td>0.4</td>
<td>0.0</td>
<td>b/1.8</td>
</tr>
<tr>
<td>Cocaine/AMP</td>
<td>1</td>
<td>0.4</td>
<td>0.0</td>
<td>650/–</td>
</tr>
<tr>
<td>Cocaine/bromazepam</td>
<td>1</td>
<td>0.4</td>
<td>0.0</td>
<td>b/18.8</td>
</tr>
<tr>
<td>Cocaine/diazepam</td>
<td>1</td>
<td>0.4</td>
<td>0.0</td>
<td>b/141/–</td>
</tr>
<tr>
<td>Cocaine/diethylpropion</td>
<td>1</td>
<td>0.4</td>
<td>0.0</td>
<td>135/–</td>
</tr>
<tr>
<td>Cocaine/MPH</td>
<td>1</td>
<td>0.4</td>
<td>0.0</td>
<td>b/18.8</td>
</tr>
<tr>
<td>Cocaine/morphine</td>
<td>1</td>
<td>0.4</td>
<td>0.0</td>
<td>4.6/–</td>
</tr>
<tr>
<td>Cocaine/tramadol</td>
<td>1</td>
<td>0.4</td>
<td>0.0</td>
<td>2.6/0.9</td>
</tr>
<tr>
<td>AMP/codeine</td>
<td>1</td>
<td>0.4</td>
<td>0.0</td>
<td>7.4/–</td>
</tr>
<tr>
<td>Femproporex/bromazepam</td>
<td>1</td>
<td>0.4</td>
<td>0.0</td>
<td>–/4.0</td>
</tr>
<tr>
<td>Femproporex/tramadol</td>
<td>1</td>
<td>0.4</td>
<td>0.0</td>
<td>–/93.1</td>
</tr>
<tr>
<td>AMP/clonazepam</td>
<td>1</td>
<td>0.4</td>
<td>0.0</td>
<td>–/21.7/2.8</td>
</tr>
<tr>
<td>AMP/clonazepam/diazepam</td>
<td>1</td>
<td>0.4</td>
<td>0.0</td>
<td>–/16/0.4</td>
</tr>
<tr>
<td>Cocaine/clonazepam/diazepam</td>
<td>1</td>
<td>0.4</td>
<td>0.0</td>
<td>1.4/1.65/1.9</td>
</tr>
<tr>
<td>Total positive samples</td>
<td>236</td>
<td>100</td>
<td>10.6</td>
<td></td>
</tr>
</tbody>
</table>

METH, methamphetamine; MDMA, 3,4-methylenedioxyamphetamine.
a Femproporex, METH and MDMA metabolize to AMP [37].
b ≥LOD = LLOQ.
c Expressed as diazepam.

11.9% of positive samples. Diazepam was the most detected benzodiazepine (39.3% of the positive samples containing this class), but lorazepam was found at the highest level (31.7 ng/mL). Cocaine and metabolites were detected in most positive samples (129, 54.7%), corresponding to 5.8% of all samples, and in 77.2% of the 22 samples containing drugs from different chemical classes. Cocaine levels reached 1165 ng/mL, lower than the levels found by Wylie et al. [17] in oral fluid samples from English drivers (4–11,110 ng/mL, mean of 1001 ng/mL). THC and/or cannabionol were found in 24 samples (1.1% of all samples, 10.2% of positive samples), from which six samples with cocaine or its metabolite EME. Opioids were found in 8 samples (0.4% of all samples, 3.4% of
positive samples), half in combination with drugs from other classes.

A national survey conducted in 2005 by the Brazilian Information Center on Psychotropic Drugs (CEBRIED) reported that 1.9% of the 7939 interviewed individuals (12 years or older) declared having used cannabis in the last month, 1.3% benzodiazepines, 0.5% cocaine/crack, 0.3% amphetamines and 0.3% opiates [39]. The results found in our study show that benzodiazepines (alone or in combination) and opioid use by Brazilian highway drivers reflects the use by the general population. However, the use rate of cocaine and amphetamines by the drivers in this study was higher than that by the general population. These drugs are stimulants of the central nervous system, delivering to the driver the desired effect of decreased fatigue, increased alertness, and combating sleep [40]. These results were also higher than those found by Yonamine [11] in the state of São Paulo, where 1.2% of the 559 oral fluid samples from truck drivers were positive for amphetamines or cocaine. In a recent study conducted in the Brazilian Southeast region, 9.3% of the urine samples from truck drivers were positive for PAD, with 5.8% positive for amphetamine, mainly fenproporex, 2.2% for cocaine and 1.1% for cannabis [12].

The prevalence of driving under the influence of drugs (DUIDs) has been demonstrated in several studies worldwide, showing a different profile from what is found in Brazil. In Australia, a study conducted with 853 oral fluid samples found a much higher incidence of positive samples, with 14% for opioids/metabolites, 8% for cocaine/metabolites, 8% for benzodiazepines and 1.5% of ketamine [24]. In the United States, the national roadside survey conducted in 2007 with 7719 oral fluid samples showed THC (8.6%) and cocaine (3.9%) as the most commonly detected drugs in nighttime drivers [41]. Jerde et al. [42] found a higher prevalence of benzodiazepines, diazepam/nordiazepam and codeine in samples collected on working days as compared with weekend samples in Norway. In our study, due to logistic and safety issues, sample collection took place only on Fridays and Saturdays (12 pm to 12 am), so this comparison could not be performed.

Considering amphetamine a metabolite of legal amphetamines, illicit drugs (MDMA, methamphetamine, cocaine and cannabinoids) were present in 7.7% of all samples analyzed in our study, 72.9% of positive samples. In Norway, the profile was inverse, with three times more samples containing prescribing drugs (3.4%) than illicit drugs (1.1%: THC, amphetamines and cocaine) [42].

The results shown in Table 3 should be interpreted critically against the limitations of the analytical method developed in this study, mainly regarding the low recovery for some analytes, such as diethylpropion and benzoylecgonine (less than 6% recovery), which might have led to an underestimation of the number of positive samples. Another limitation was the lack of 6-acetylmorphine, the specific metabolite of heroin [37], although the prevalence of this illicit drug in Brazil is not significant [39]. This study also did not include the crack pyrolysis products anhydroecgonine and anhydroecgonine methyl-ester in the method, which would enable the detection of crack users. The widespread use of crack cocaine has become a public health issue in Brazil in recent years [43,44].

4. Conclusion

Obtaining a good recovery for analytes from different chemical classes while maintaining good sensitivity is a challenge that most authors have encountered in method development. This paper described a LC–MS/MS method for the simultaneous determination of 32 compounds (amphetamine, benzoylecgonine, cocaine, cannabis, opioids, ketamine and m-CPP and their respective metabolites) in oral fluid samples. The method proved to be simple and useful for the analysis of a large amount of samples, and showed to be quantitative for 24 compounds, including all benzodiazepines and cocaine. This is first oral fluid LC–MS/MS method to include fenproporex, diethylpropion and m-CPP. About 10% of the 2235 oral fluid samples collected from Brazilian drivers showed to be positive for at least one drug investigated, mostly from cocaine and amphetamine users. The results indicate a need for preventive measures aimed at reducing the use of psychoactive drugs by drivers in Brazil, which will certainly have a positive impact on decreasing the country’s highway death rates.

Acknowledgments

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References