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Detection of phencyclidine in human oral fluid using solid-phase extraction and liquid chromatography with tandem mass spectrometric detection

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Abstract

An analytical procedure for the determination of phencyclidine in oral fluid has been developed and validated using liquid chromatography with tandem mass spectral detection, following initial screening with enzyme linked immunosorbent assay. The oral fluid samples were collected using the Quantisal ™ device, and any drugs present were quantified using mixed mode solid-phase extraction followed by mass spectrometric detection in positive atmospheric pressure chemical ionization mode. For confirmation, two transitions were monitored and one ratio determined, which had to be within 20% of that of the known calibration standard. The monitoring of the qualifying transition and requirement for its presence within a specific ratio to the primary ion has the potential of limiting the sensitivity of the assay, however, the additional confidence in the final result as well as forensic defensibility were considered to be of greater importance. The limit of quantitation was 5 ng/mL; the intra-day precision of the assay ($n = 5$) was 3.04%; inter-day precision 3.35% ($n = 5$) at a concentration of 10 ng/mL. The accuracy was determined at four concentrations (5, 10, 20 and 40 ng/mL) within the linear range of the assay. The percentage recovery of phencyclidine from the oral fluid collection pad was 81.7% ($n = 6$). The methods were applied to both proficiency specimens and to samples obtained during research studies in the USA.

Keywords: Oral fluid; Phencyclidine; LC/MS/MS

1. Introduction

Oral fluid is increasing in popularity as an alternative matrix to blood or urine for standard drug testing due to its ease of collection, difficulty of adulteration and improving sensitivity of analytical techniques. Phencyclidine (PCP) is included in the proposed United States Federal regulations for saliva drug testing in the workplace, and the suggested cut-off concentration is 10 ng/mL of neat oral fluid. Surprisingly, there are no published procedures for the determination of PCP in oral fluid, using liquid chromatography with tandem mass spectrometry, however, there is one method for its analysis in rat serum [1]. Other methods for the determination of PCP in blood [2], urine [3], hair [4] and meconium [5] have been reported, which incorporate the more standard gas chromatography–mass spectrometry instrumentation, usually found in forensic laboratories.

There are publications describing the analysis of various other drugs of abuse in oral fluid using LC/MS/MS in APCI mode, in a similar manner to our approach however, many of these procedures monitor only one transition in the multiple reaction-monitoring mode (MRM). Recently, several authors have focused on the need for the monitoring a second transition, allowing the ratio between the abundance of the primary and secondary ions to be calculated, and establishing more confidence in the final result. Maralikova and Weinmann [6] noted that guidelines for confirmatory analysis using LC/MS/MS have not yet been established, and suggest that the monitoring of at least two transitions is required to provide sufficient identification of drugs.

One of the main issues with the quantitation of drugs in oral fluid is the difficulty of collection in terms of specimen volume. Many of the currently available devices do not give an indication of how much oral fluid is collected, thereby rendering any

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quantitative results meaningless without further manipulation in the laboratory [7]. Further, devices incorporating a pad or material for the saliva collection do not always indicate how much of each drug is recovered from the pad before analysis, again calling into question any quantitative result. The drug concentration reported is dependent on the collection procedure used [8]. This work employed the Quantisal ™ oral fluid collection device, which collects a known amount of neat oral fluid. The efficiency of recovery of PCP from the collection pad into the transportation buffer was determined, in order to increase confidence in the quantitative value. The stability of the drugs in the buffer at room temperature and at 4 °C was studied, as well as the stability of extracted oral fluid specimens.

We have validated a procedure for the determination of PCP in oral fluid, which provides forensic defensibility for the generated result in terms of specimen volume, drug recovery from the collection pad and LC/MS/MS with two monitored transitions. The method was applied to specimens received into our laboratory from proficiency programs and research studies.

2. Experimental

2.1. Oral fluid collection devices

Quantisal ™ devices for the collection of oral fluid specimens were obtained from Immunalysis Corporation (Pomona, CA). The devices contain a collection pad with a volume adequacy indicator, which turns blue when one milliliter of oral fluid (±10%) has been collected. The pad is then placed into transport buffer (3 mL), allowing a total specimen volume available for analysis of 4 mL (3 mL buffer + 1 mL oral fluid). This is specifically advantageous in cases where the specimen is positive for more than one drug and the volume of specimen available for analysis may be an issue. The oral fluid concentration is diluted 1:3 when using Quantisal ™ collection devices, and drug concentrations detected were adjusted accordingly.

2.2. Standards and reagents

The phencyclidine direct ELISA kit (Catalog #208) was obtained from Immunalysis Corporation (Pomona, CA) and used for screening the oral fluid samples. For confirmatory procedures, penta-deuterated internal standard (phencyclidine-d5) as well as unlabelled drug standard was obtained from Cerilliant (Round Rock, TX). Solid-phase extraction columns (Clin II, 691-0353T) were obtained from SPEWare, (San Pedro, CA). All solvents were HPLC grade or better, and all chemicals were ACS grade.

2.3. Calibrators

For the chromatographic calibration standards, a working solution for the deuterated internal standard was prepared in methanol at a concentration of 250 ng/mL. Unlabelled drug standard was prepared in methanol at the same concentration. All the working solutions were stored at −20 °C when not in use. For each batch, four calibration standards were prepared in synthetic oral fluid (1 mL) then transportation buffer from the Quantisal ™ collection device was added (3 mL). A synthetic oral fluid matrix, which matched the immunoassay responses of three human negative oral fluid samples was prepared, comprising 25 mM phosphate buffered saline (pH 7.0), 30 mM sodium bicarbonate, 0.1% albumin, amylase and 0.1% Proclin 300 as a preservative. Synthetic oral fluid was used as opposed to authentic drug free saliva primarily because of the amount required in order to carry out all the experiments. The effect of real oral fluid on the drugs compared to the effect in synthetic material is minimized during 1:4 dilution with transportation buffer. Drug concentrations of 2.5, 5, 10, 20, 40, 100 and 200 ng/mL of neat oral fluid equivalents were prepared (internal standard concentration: 20 ng/mL).

2.4. Screening assay

Enzyme linked immunosorbent assays (ELISA) technology is based upon the competitive binding to antibody of enzyme labeled antigen and unlabeled antigen in proportion to their concentration in the reaction well. The oral fluid specimens were screened at a concentration of 10 ng/mL for phencyclidine. A standard curve consisting of a drug free negative oral fluid specimen, and drug free oral fluid specimens spiked at 50% and 200% of the recommended cut-off concentrations was analyzed with every batch.

The optimal sample size as suggested by the manufacturer was 10 μL. The sample volume was pipetted directly from the collection device into the microplate. Specimens screening positively using ELISA, were carried forward to confirmation using the described procedure.

2.5. Sample preparation for chromatographic analysis

An aliquot (1 mL) from the Quantisal ™ collection device, equivalent to 0.25 mL of neat oral fluid equivalents was removed and internal standard was added (20 μL); 0.1 M sodium phosphate buffer (pH 6.0; 1 mL) was added to each calibrator, control or oral fluid specimen. Solid-phase mixed mode extraction columns (Clin II, 691–0353T) were placed into a positive pressure manifold. Each column was conditioned with methanol (2 mL), and 0.1 M phosphate buffer (pH 6.0; 2 mL). The samples were allowed to flow through the columns, and then the columns were washed with deionized water (1 mL), 0.1 M acetate buffer (pH 4; 1 mL), methanol (1 mL) and ethyl acetate (1 mL). The columns were allowed to dry under nitrogen pressure (30 psi; 2 min). The drugs were finally eluted using freshly prepared ethyl acetate: ammonium hydroxide (98:2 v,v; 2 mL). The extracts were evaporated to dryness under nitrogen and reconstituted in 20 mM ammonium formate (pH 6.4): methanol (70:30; v,v) (40 μL).

2.6. Liquid chromatography –tandem mass spectrometry (LC/MS/MS)

A1200 Series liquid chromatograph pump coupled to a 6410 triple quadrupole mass spectrometer, operating in pos-

itive atmospheric pressure chemical ionization mode (APCI) mode was used for analysis (Agilent Technologies, Santa Clara, CA). The liquid chromatographic column was also supplied by Agilent technologies, and was a Zorbax Eclipse XDB C18 (4.6 × 50 mm × 1.8 μm). The column temperature was held at 40 °C and the injection volume was 5 μL. The mobile phase consisted of 20 mM ammonium formate (pH 6.4, solvent A) and methanol (solvent B). Initially, the mobile phase composition was 75% A: 25% B at a flowrate of 0.9 mL/min. After 1.5 min, the flowrate was changed to 1 mL/min for the remainder of the run. After 1.5 min, the percentage of solvent B was 30%, after 4.5 min 55%, after 5 min 60% and finally after 7 min, 75%. The gas temperature was 350 °C, the gas flow was 5 L/min and the nebulizer pressure was 50 psi. Nitrogen was used as the collision gas and the capillary voltage was 4500 V.

The molecular weight of PCP is 243 (Fig. 1); deuterated (d5) PCP has a molecular weight of 248. Two transitions were selected and optimized for each drug using flow injection analysis. For deuterated (d5) PCP, the parent ion *m/z* 249.3 (M + 1) was fragmented at a voltage of 40 V with optimal collision energy of 15 V to *m/z* 164.3. For unlabelled PCP, two transitions were monitored: *m/z* 244.3–91.2 and *m/z* 244.3–86.2. For both transitions, the optimal fragment voltage was 5 V and collision energy 25 V. For the deuterated standard, the intensity and consistency of the *m/z* 249.3–164.3 transition was sufficient for use in the assay. However, the corresponding transition for non-deuterated PCP, from *m/z* 244.3 to 159 was weak. The transition from 249.3 to 96.1 was also adequate for use in the assay as shown in Fig. 2. The transitions from *m/z* 244.3 to 91.2 and *m/z* 244.3–86.2 were found to be much more intense, and were selected for the validation of the assay. The ratio of the qualifying transition to the quantifying transition was determined at the proposed cut-off concentration of 10 ng/mL.

2.7. Data analysis

Calibration using deuterated internal standard was calculated using linear regression analysis over a concentration range of 5–200 ng/mL. Peak area ratios of the target analyte and the internal standard were calculated using Mass Hunter software (Agilent). The data were fit to a linear least-squares regression curve with a 1/*x* weighting and were not forced through the origin.

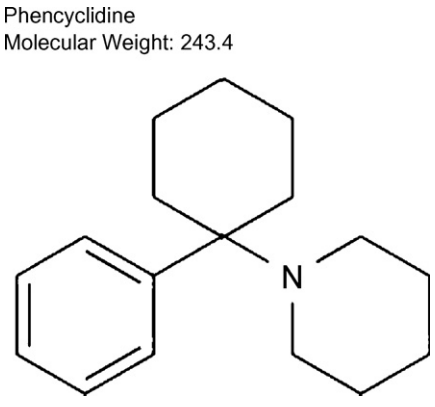

Fig. 1. Structure of phencyclidine (PCP).

2.8. Selectivity

Drug free oral fluid specimens were obtained from three volunteers and extracted and analyzed according to the described procedures in order to assess interference from extraction or matrix, or potential ion suppression. Ion suppression is caused by competition among ions (from the analyte, matrix, salts, mobile phase etc.) for the limited number of excess charge sites on the generated liquid droplets during ESI. Since the ionization for APCI occurs in the gaseous phase, there is less competition, so the phenomenon is not as prevalent. In fact, Liang et al. [9] investigated nine drugs in both modes, reporting that ESI suppressed the ionization response of all the drugs. In contrast, APCI caused seven of the drugs, and their co-eluting isotope labeled internal standards to enhance each other's ionization response. Further, following the approach of Liang et al, in order to minimize potential suppressive effects, an isotope-labeled internal standard was employed at a concentration within the range of the calibration curve and solid-phase extraction was used to limit matrix effects. Mei et al [10] reported that ion suppression is of greater concern in ESI than APCI, but the effects were not only ionization mode dependent, but also instrument dependent.

To monitor potential ion suppression, an unextracted drug standard at a concentration of 10 ng/mL was prepared as well as drug free matrix extracts and negative controls (extracts containing only internal standard).

Interferences from commonly encountered drugs were added to the drug free oral fluid specimens and subjected to the same extraction and analysis procedures. The following drugs were analyzed using the described procedures at a concentration of 10,000 ng/mL: cocaine, benzoylecgonine, cocaethylene, norcocaine, morphine, 6-acetylmorphine, codeine, hydrocodone, hydromorphone, oxycodone, oxymorphone, tramadol, fentanyl, gamma-hydroxybutyrate (GHB), tetrahydrocannabinol (THC), 9-carboxy-THC, amphetamine, methamphetamine, methylenedioxymethamphetamine (MDMA), methylenedioxyamphetamine (MDA), methylenedioxyethylamphetamine (MDEA), carisoprodol, methadone, diazepam, nordiazepam, oxazepam, alprazolam, chlordiazepoxide, bromazepam, temazepam, lorazepam, flurazepam, nitrazepam, triazolam, amitryptiline, nortriptyline, imipramine, protriptyline, doxepin, nordoxepin, trimipramine, secobarbital, pentobarbital, butalbital, and phenobarbital. Various designer drugs derived from PCP have recently been reported, which may fragment to similar ions in the APCI source; however, the initial monitored ion (244.3) would not be found in these particular derivatives [11].

2.9. Linearity and sensitivity

The linearity of the assays was established with seven calibration points, excluding the drug free matrix. The sensitivity of the method was determined by establishing the lower limit of quantitation (LLOQ) defined as the lowest concentration detectable with a signal to noise (S:N) ratio of at least 10 and retention time within 0.2 min of the calibration standard.

2.10. Accuracy and precision

The accuracy of the procedure was determined over six replicates at four concentrations (5, 10, 20 and 40 ng/mL). Accuracy was calculated as (mean measured concentration–fortified concentration) divided by the fortified concentration × 100%.

Inter and intra-day precision of the assays was determined at the calibration point of 10 ng/mL. Intra-day data were obtained from five analyses performed on one day; inter-day data were obtained by analyzing a total of five specimens over 5 days.

2.11. Extraction efficiency

One of the main issues associated with oral fluid analysis is recovery of drug from a collection pad; therefore the efficiency of PCP extraction from the collection device was determined. Synthetic oral fluid was fortified with PCP at the concentration of 10 ng/mL.

A collection pad was placed into the fluid until the volume adequacy indicator turned blue showing that 1 mL (±10%) of oral fluid had been absorbed. The pads were then placed into the Quantisal ™ buffer (3 mL), capped, and allowed to remain at room temperature overnight, to simulate transportation to the laboratory. The following day, the pads were removed after separation from the stem, and an aliquot (1 mL) of the specimen was analyzed. The procedure was repeated six times.

2.12. Stability

The stability of PCP in the oral fluid collection device during transportation was assessed. Three Quantisal ™ devices fortified with PCP at a concentration of 10 ng/mL were sent via common courier to the East Coast of the USA and back to our facility in California. The time between shipment and return receipt was 96 h. Temperature monitors in the courier bags indicated the minimum temperature during shipment was 20.6 °C; the maximum temperature was 33.9 °C. The concentration of PCP was measured and compared to reference specimens stored for the same period of time at 4 °C. The stability of the drug extracts at a concentration of 10 ng/mL was determined by allowing the autosampler vials to remain in the liquid chromatographic chamber for 48 h after which time they were re-analyzed. The unit was maintained at 7 °C. The responses were compared to those achieved on the first day of analysis.

2.13. Application to authentic specimens

As part of various on-going research studies, our laboratory receives oral fluid specimens for research purposes as well as proficiency specimens.

3. Results and discussion

3.1. Method validation

The chromatographic procedure developed for PCP was validated according to accepted protocols. The limit of quantitation was 5 ng/mL and was determined as described in the Section 2 (Experimental section). Linearity was obtained with an average correlation coefficient for all the drugs of >0.99 over the range from 5 to 200 ng/mL of oral fluid. The mean correlation for the calibration curve was $r^2 = 0.99644$ ($n = 6$) with an average slope equation of $y = 0.1531x - 0.2032$, where x is the concentration of PCP and the relative response, y, is the peak area response of the drug/peak area response of the internal standard. For quantitation, the transition from *m/z* 244.3 to 91.2 was used; *m/z* 244.3–86.2 as the qualifying transition. The ratio of the intensity of the qualifying transition to the intensity of the quantifying transition was 74.2% at the concentration of 10 ng/mL. For subsequent analyses, the allowable qualifying ratio for the intensity of the second transition was 59.6–89.5% (±20%).

3.2. Recovery and interference

The recovery of PCP from the collection pad using the Quantisal ™ device was determined to be 81.67% (S.D. 1.17; $n = 6$). Oral fluid specimens collected from drug free individuals showed no interference with any of the assays, which was not unexpected, since it is unlikely these drugs are similar to endogenous substances in oral fluid. For exogenous interferences, commonly encountered drugs of abuse were studied as described in the Section 2 (Experimental section). No chromatographic interference was observed in the channels of these transitions. Since the oral fluid is diluted during in collection, a deuterated internal standard is employed, then the drugs

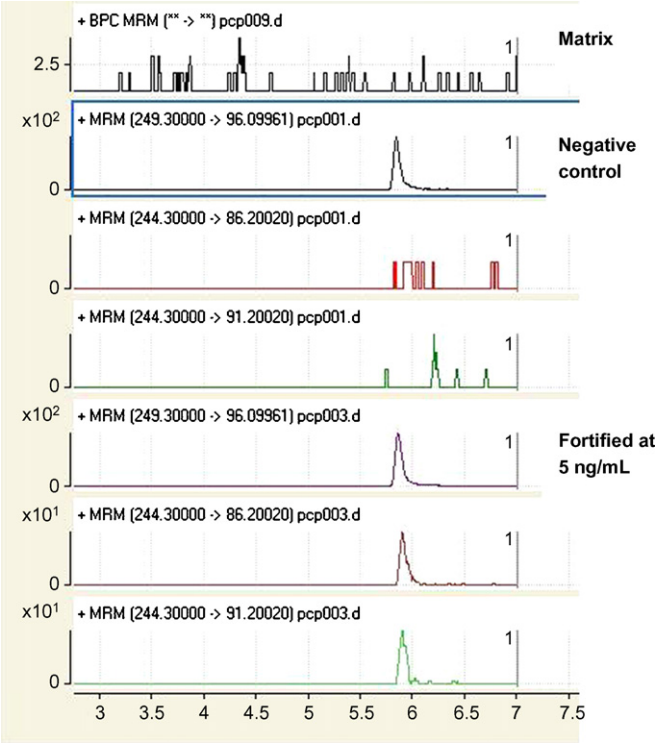

Fig. 2. Ion chromatograms of blank matrix, negative control and oral fluid specimen fortified with 5 ng/mL (LLOQ).

are extracted using a specific solid-phase procedure, significant ion suppression was not observed. Chromatograms from a drug free matrix (blank), drug free matrix with internal standard added (negative control) and an oral fluid fortified with 5 ng/mL of PCP (lower limit of quantitation) are shown in Fig. 2.

3.3. Precision, accuracy and stability

The validation data for the assay is shown in Table 1. The procedure was very accurate, with a maximum variation of −6.5% from the fortified level at the cut-off concentration. Inter-day (between day) and intra-day (same day) precision of the assay was determined using replicate analyses as described. The inter-day precision was 3.34% ($n = 5$); intra-day precision was 3.05% ($n = 5$). Finally, the stability of the drugs in the collection system during transportation, and the stability of the extracts were assessed.

Following transportation, an average of 8.9 ng/mL of PCP was measured in the specimens compared to 9.4 ng/mL in the specimens from cold storage, a loss of 5.3% during shipment.

The extracts were stable for at least 2 days when kept in the instrument rack inside the auto sampler, which was maintained at 4 °C. There was less than a 5% difference in the quantitation of the extracts after 48 h.

3.4. Authentic specimens

The procedures were applied to proficiency specimens received into the laboratory. The performance was excellent, with all quantitation being within 10% of the group mean identified by the program administrators. An example of an authentic oral fluid specimen at a concentration of 38 ng/mL is shown in Fig. 3.

Table 1
Validation data for determination of PCP in oral fluid

Nominal concentration	5 (ng/mL)	10 (ng/mL)	20 (ng/mL)	40 (ng/mL)
Assay run #				
1	4.7	9.5	21	39
2	5.4	9.0	19	40
3	5.3	9.2	19	40
4	5.6	9.2	18	38
5	5	9.8	18	42
6	5	9.4	21	39
Mean (ng/mL)	5.1	9.3	19.3	39
Accuracy (%)	3.3	−6.5	−3.3	−0.83

	Intra-day ($n = 5$) concentration (ng/mL)	Inter-day ($n = 5$) concentration (ng/mL)
	10	9.5
	10.8	9.0
	10.7	9.2
	10.7	9.2
	10.5	9.8
Mean (ng/mL)	10.54	9.34
S.D.	0.32	0.31
CV (%)	3.04	3.35

Accuracy: controls fortified at four concentrations. Precision: controls fortified at 10 ng/mL.

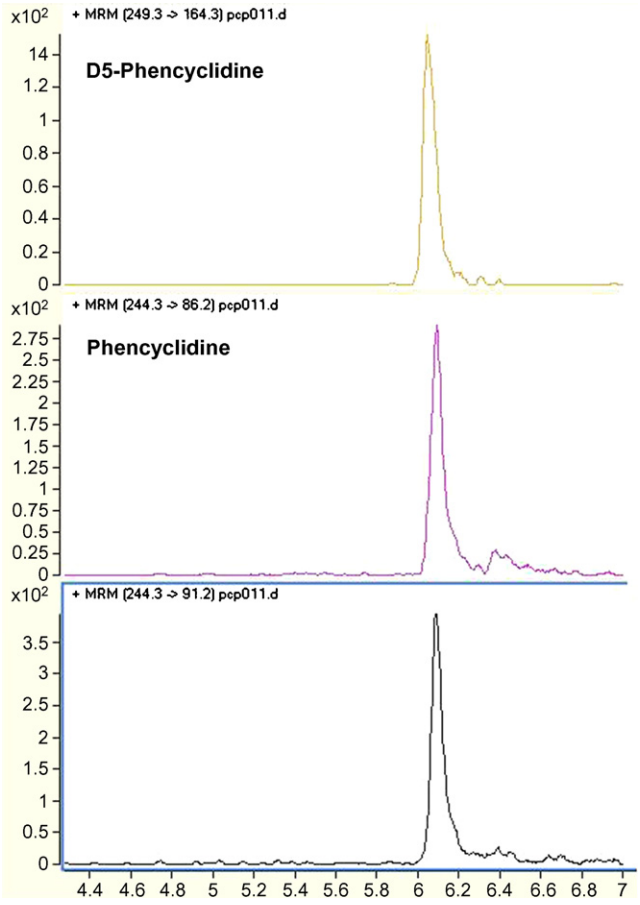

Fig. 3. PCP detected in an authentic oral fluid specimen (38 ng/mL).

4. Conclusions

The determination of phencyclidine in oral fluid is described. The LC/MS/MS procedure is reproducible, robust and precise. The assay includes the monitoring of a qualifying transition and calculation of a ratio, required to be within 20% of that of a known calibration standard in order for definitive identification to be made. The method is easily incorporated into routine laboratory testing.

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