Detection of Phencyclidine in Human Oral Fluid Using Solid Phase Extraction and Liquid Chromatography with Tandem Mass Spectrometric Detection

Application Note

Forensic Toxicology

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Abstract

An analytical procedure for the determination of phencyclidine in oral fluid has been developed 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 intraday precision of the assay was 3.04% (n = 5); interday precision was 3.35% (n = 5). 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.

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 the 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 (LC/MS/MS). However, there is one method for its analysis in rat plasma [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.

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 to monitor 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 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 [6].

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 quantitative results meaningless without further manipulation in the laboratory [7]. Furthermore, 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].

The work presented here employed the Quantisal™ oral fluid collection device, which collected a known amount of neat oral fluid. The recovery efficiency 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 verified a procedure for the determination of PCP in oral fluid that 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 is applied to specimens received into our laboratory from proficiency programs and research studies.

The structure of PCP is shown in Figure 1.

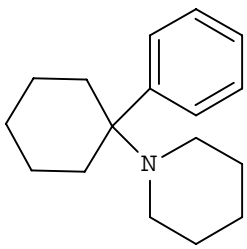

Figure 1. Structure of phencyclidine (PCP).

Experimental

Sample Preparation

Oral Fluid Collection Devices
Quantisal™ devices for the collection of oral fluid specimens are 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%) is 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 are adjusted accordingly.

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, pentadeuterated internal standard (phencyclidine-d5) as well as unlabeled drug standard were 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.

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. Unlabeled 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). Drug concentrations of 5, 10, 20, and 40 ng/mL of neat oral fluid equivalents were prepared (internal standard concentration: 20 ng/mL).

Screening Assay
Enzyme linked immunosorbent assay (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 according to the manufacturer's instruc-

tions, which recommended cut-off concentrations of 10 ng/mL for phencyclidine; of neat oral fluid equivalents. 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.

Sample Preparation for Chromatographic Analysis

An aliquot (1 mL) from the Quantisal™ collection device, equivalent to 0.25 mL of neat oral fluid, was removed and internal standard (20 µL) was added. 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 70:30 v/v of 20 mM ammonium formate (pH 6.4) and methanol (40 µL).

Analytical Procedure

Instrument:	Agilent 1200 Series RRLC; 6410 LC Triple Quadrupole Mass Spectrometer

LC Conditions:

Column:	ZORBAX Eclipse XDB C18, 4.6 mm x 50 mm x 1.8 µm, (p/n 822795-902)
Column temperature:	40 °C
Solvent flow rate:	0.6 mL/min
Mobile phase:	A = 20 mM ammonium formate, pH 6.4 B = methanol
Injection volume:	5 µL

Gradient:

Time (minutes)	%B	Flow rate (mL/min)
0	25	0.9
1.5	30	0.9
4.5	55	1
5	60	1
7	75	1

Stop time = 7 min; Post time = 3 min

MS Conditions:

Operation:	Positive APCI mode
Gas temperature:	350 °C
Gas flow (N_2):	5 L/min
Nebulizer pressure:	50 psi
Capillary voltage:	4500 V

The multiple reaction monitoring (MRM) transitions are shown in Table 1. Derived retention times are also given. For all transitions the first quadrupole, for the precursor ion, is operated at wide resolution, or full width half maximum (FWHM) equal to 2.5 amu. The last quadrupole, for the product ions, is operated at unit resolution, or FWHM equal to 0.7 amu. Finally, the dwell time for each transition is 75 msec.

Table 1. Multiple Reaction Monitoring (MRM) Transitions for Phencyclidine and Its Deuterated Analog (D5), Used as the Internal Standard (IStd)

Compound	RT (min)	MRM transition	Frag (V)	CE (V)
PCP	6.1	244.3 > 91.2 (86.2)	40	25 (25)
PCP-D5	6.1	249.3 > 164.3	40	15

* ( ) qualifier ions; qualifier ratios must be within 20% of calibration point

Results and Discussion

Data Analysis

Calibration using deuterated internal standard was calculated using linear regression analysis over a concentration range of 5 to 40 ng/mL. Peak area ratios of the target analyte and the internal standard were calculated using MassHunter software (Agilent). The data were fit to a linear least-squares regression curve with no weighting and was not forced through the origin.

Method Development

The development of a simple LC/MS/MS assay for the detection of phencyclidine in oral fluid is reported. While these drugs have been detected in oral fluid, the increasing utility of LC/MS/MS in

laboratories makes development of confirmatory procedures necessary and timely. The monitoring of a second qualifying ion is reported for the first time for the determination of PCP in oral fluid analysis and is necessary for the improved confidence in the identification of the analyte.

Method Verification

The chromatographic procedure developed for PCP was verified according to accepted protocols. The limit of quantitation was 5 ng/mL and was determined as described in the Experimental section. Linearity was obtained with an average correlation coefficient for all the drugs of > 0.99 over the dynamic range from 5 to 40 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, where x = concentration of PCP and the relative response, y, = peak area response of the drug/peak area response of the internal stan-dard. An example of one of the calibration curves is shown in Figure 2.

Method of Confirmation

Two product ions from fragmentation of PCP were monitored. The most intense (*m/z* = 91.2) was used for quantitation. The least intense of the two (*m/z* = 86.2) was used as a qualifier for ion ratio confirmation. That is, the ratio of the two peak areas must have been consistent, and within a tolerance of ± 20%, to be considered acceptable. The allowable qualifying ratio for the intensity of the second transition is 59.6% to 89.5% (± 20% of 0.74) and applied across all batches. An example at the lowest calibration level of 5 ng/mL is shown in Figure 3.

Recovery and Interference

The recovery of PCP from the collection pad using the Quantisal™ device was determined to be 81.67% (SD 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 that these drugs

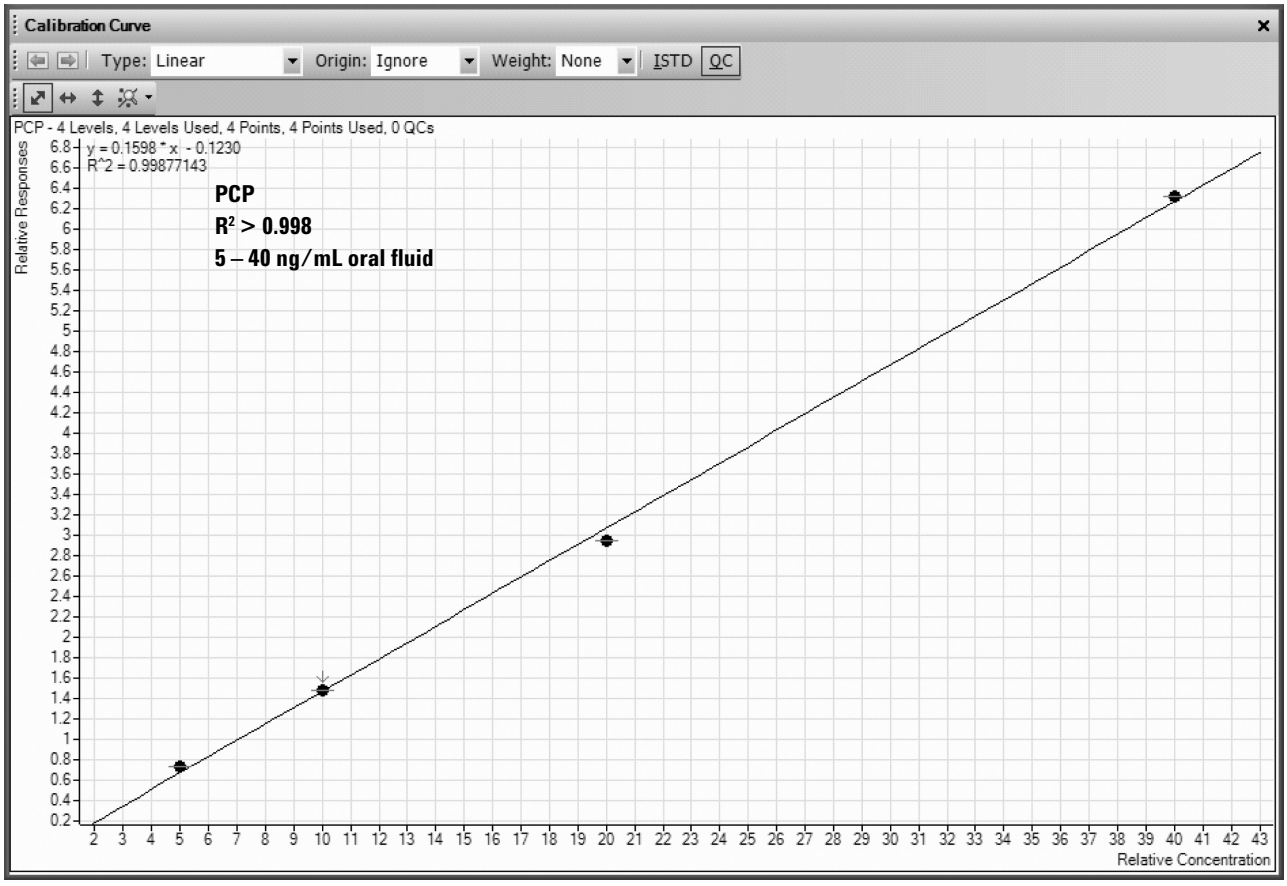

Figure 2. Linearity of PCP.

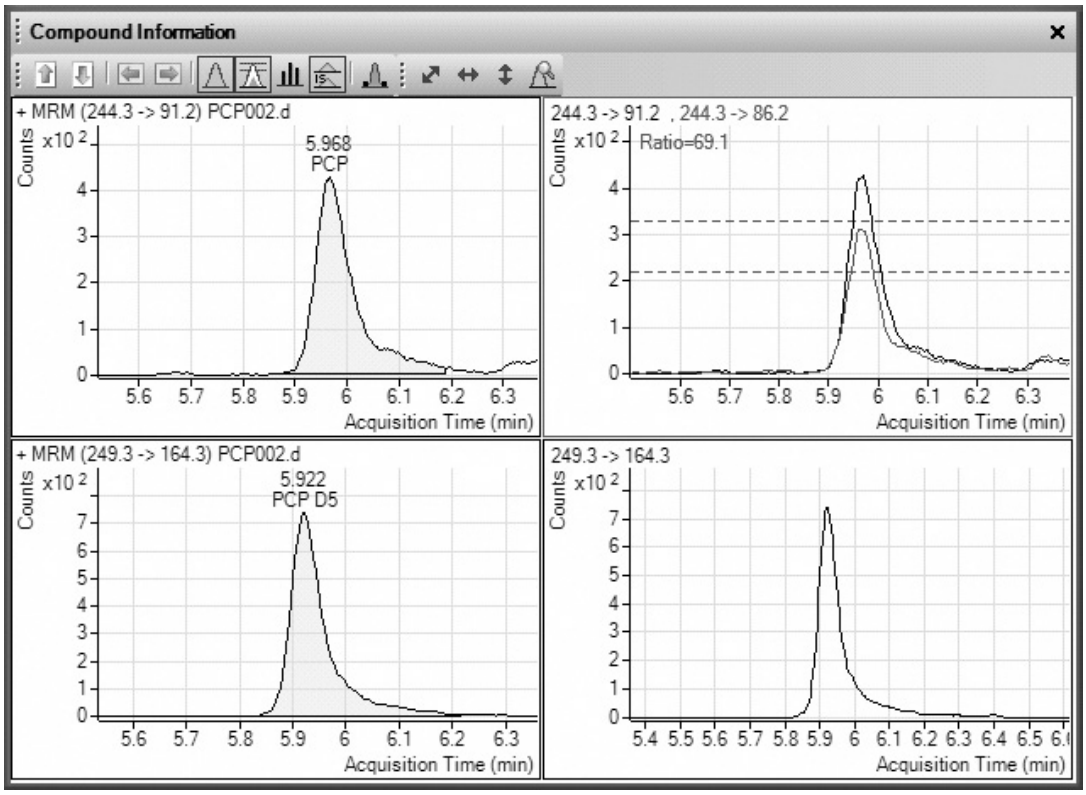

Figure 3. Confirming the presence of PCP using quant/qual ion ratios. In this example, the ratio of the lowest calibration level of 5 ng/mL is 0.69, which is within 20% of 0.74.

are similar to endogenous substances in oral fluid. For exogenous interferences, commonly encountered drugs of abuse were studied as described in the Experimental section. No chromatographic interference was observed in the channels of these transitions. Since the oral fluid was diluted during collection and the drugs are extracted using a specific solid-phase procedure, ion suppression of any significance was not observed.

Precision, Accuracy, and Stability

The accuracy of the assay was determined as described and the results are shown in Table 2. The procedure was very accurate, with a maximum variation of –6.5% from the fortified level at the cut-off concentration. The interday (between-day) and intraday (same-day) precision of the assay was determined using replicate analyses as described. The interday precision was 3.35% (n = 5); intraday precision was 3.04% (n = 5). Finally, the stability of the drugs in the collection system and the stability of the extracts were assessed. The extracts were stable for at least 2 days when kept in the instrument rack inside the autosampler, which was maintained at 4 °C. There was less than a 5 % difference in the quantitation of the extracts after 48 hours.

Table 2. Interassay Accuracy from Six Analytical Runs

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	19.8	40
Accuracy (%)	3.3	–6.5	–3.3	–0.83

Table 3. Intraday and Interday Reproducibility Monitoring the 10 ng/mL Control Level

Nominal concentration	Interday (n = 5)	Intraday (n = 5)
	9.5	10
	9.0	10.8
	9.2	10.7
	9.2	10.7
	9.8	10.5
Mean (ng/mL)	9.34	0.54
Std Dev.	0.31	0.32
Accuracy (%)	3.35	3.04

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 14.7 ng/mL is shown in Figure 4.

Conclusions

The determination of PCP 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.

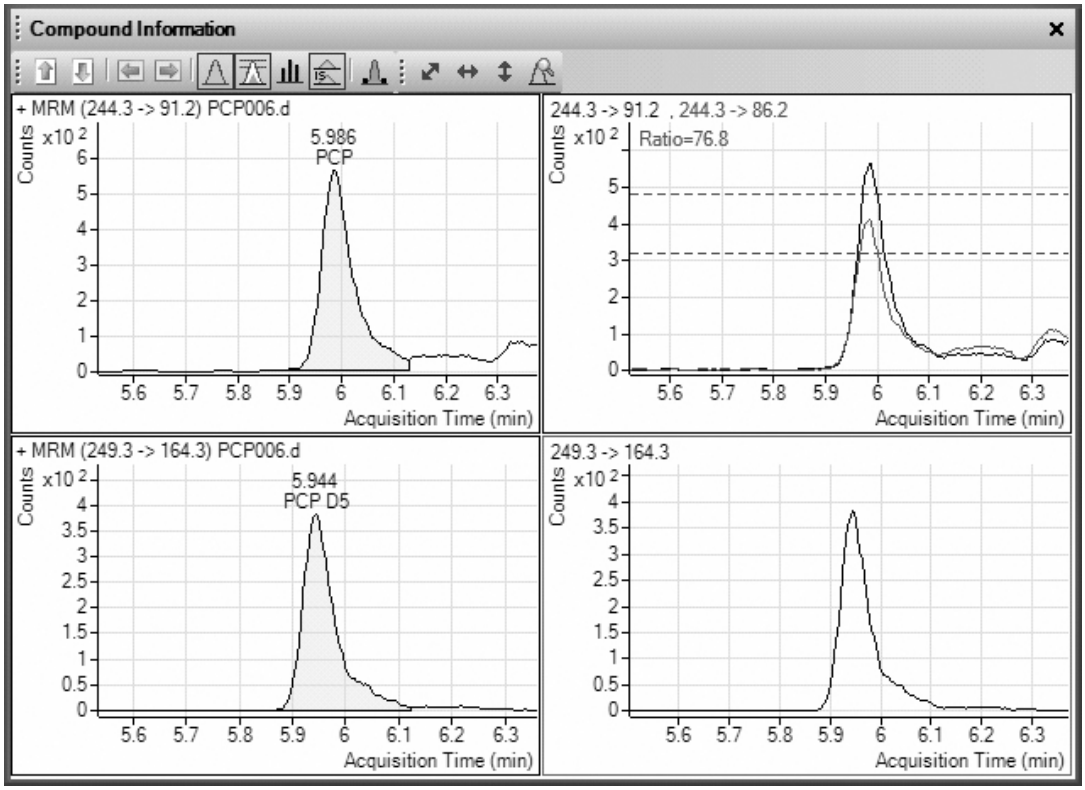

Figure 4. Confirming the presence of PCP using quant/qual ion ratios in an actual patient sample at a level of 14.8 ng/mL.

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