

Antidepressant Drugs in Oral Fluid Using Liquid Chromatography–Tandem Mass Spectrometry

Cynthia Coulter, Margaux Taruc, James Tuyay, and Christine Moore*

Immunoanalysis Corporation, 829 Towne Center Drive, Pomona, California 91767

Abstract

An analytical procedure for the determination of widely prescribed drugs for the treatment of depression and anxiety disorders, including amitriptyline, cyclobenzaprine, imipramine, dothiepin, doxepin, fluoxetine, sertraline, trimipramine, protriptyline, chlorpromazine, clomipramine, and some of their metabolites (nortriptyline, desmethyldoxepin, desipramine, desmethyltrimipramine, norclomipramine) in oral fluid has been developed and validated using liquid chromatography with tandem mass spectral detection. The oral fluid samples were collected using the Quantisal™ device, screened with enzyme-linked immunosorbent assay. Any drugs present were quantified using mixed-mode solid-phase extraction followed by mass spectrometric detection in positive electrospray ionization mode. For confirmation, two transitions were monitored, and the ratio between the two was required to be within 20% of the known calibration standard. Because of the worldwide shortage of acetonitrile, which was first reported in October 2008, the mobile phase was optimized to use methanol as the organic component. For all compounds, the lower limit of quantitation was 5 ng/mL; the intraday precision ranged from 2.9 to 8.2% ($n = 6$); interday precision from 1.5 to 6.2% ($n = 30$) at a concentration of 40 ng/mL. The percentage recovery of antidepressants from the oral fluid collection pad was calculated at a concentration of 40 ng/mL and ranged from 51.4 to 84.1% ($n = 6$). The aim of the study was to develop a confirmatory procedure for drugs in oral fluid that had been identified as presumptively positive for antidepressants and related compounds. The methods were applied to research oral fluid specimens received into our facility for testing.

Introduction

Antidepressant drugs are widely prescribed for the management of depression but are also used in the treatment of other issues, such as anxiety, eating problems, chronic pain, mi-

graines, or sleep disorders. Antidepressant drugs may also cause impairment of cognitive and psychomotor functioning relevant to driving, although this impairment is reportedly less severe in second generation antidepressants such as selective serotonin reuptake inhibitors (SSRIs) than with more traditional tricyclic antidepressants (TCAs) such as amitriptyline (1,2). Patients receiving long-term treatment with SSRI and serotonin-norepinephrine reuptake inhibitor (SNRI)-type drugs produce impaired driving performance (3). However, Brunnauer et al. (2) showed that there were actually no statistically significant differences between patients treated with TCAs and those receiving the SNRI venlafaxine yet concluded that approximately 16% of depressive patients discharged from hospital to outpatient treatment must still be considered unfit to drive. Severe depression in drivers of heavy goods vehicles was shown to be associated with an increased risk of being involved in traffic accidents (4), and cases of driving under the influence of these types of antidepressants have been reported (5). Conversely, a recent study reported that mirtazapine, a sedating antidepressant, actually increased driving safety in patients with major depressive disorders (6). Also in 2008, Iwamoto et al. (7) showed that amitriptyline significantly impaired driving performance on crowded urban roads, but paroxetine impaired neither cognitive function nor driving ability. In a separate study, the same research group recommends therapeutic monitoring of amitriptyline as a useful predictor of driving problems (8). Because most of the literature appears to point to some degree of driving impairment, particularly in long-term users of antidepressant drugs, the monitoring or detection of these substances in specimens collected at the roadside is particularly timely.

Oral fluid is increasing in popularity as a drug-testing matrix due to its ease of collection, difficulty of adulteration, and improving technology allowing for expanded drug test profiles. In areas of traffic safety, oral fluid has been studied worldwide as an alternative matrix for collection at the roadside, with major studies being performed in Europe, Australia, Canada, and the U.S.

* Author to whom correspondence should be addressed. E-mail: cmoore@immunoanalysis.com.

One of the main issues with the quantitation of drugs in oral fluid is the difficulty of adequately collecting known specimen volumes, so that a wide range of drugs can be reliably quantified. Many of the currently available devices do not give an indication of the amount of oral fluid collected, thereby rendering any quantitative results meaningless without further manipulation in the laboratory (9). Further, devices incorporating a pad or material for the oral fluid collection do not always indicate how much of each drug is recovered from the pad before analysis, which again calls any quantitative result into question. The research described in this paper uses the Quantisal™ oral fluid collection device, which collects a known amount of neat oral fluid. The efficiency of recovery of the antidepressant drugs from the collection pad into the transportation buffer was determined in order to increase confidence in the quantitative value. Further, the stability of the drugs in extracted oral fluid specimens was studied. Several publications describe the analysis of some of these drugs in plasma using liquid chromatography with tandem mass spectrometry (LC–MS–MS) with positive ion electrospray ionization (ESI) techniques. Patel et al. report on the detection of venlafaxine and its metabolite in 2008 and on the analysis of sertraline and its main metabolite in 2009 (10,11) but methods for measuring antidepressants in oral fluid are not plentiful. Concheiro et al. (12) include amitriptyline in a panel of drugs from preserved oral fluid, and de Castro et al. (2008) reported on the simultaneous determination of nine antidepressants and four metabolites in plasma and oral fluid using LC–MS–MS (13). Although our approach to the analysis was similar to the publications cited, one major difference was the incorporation of methanol into the mobile phase due to the worldwide shortage of acetonitrile, which was first reported in October 2008. Further, our procedure provided confirmation methodology for specimens screening positively for antidepressants using enzyme-linked immunosorbent assay (ELISA). Even though fluoxetine and sertraline do not cross-react in the screening assay, they are available as drug-specific screening ELISA assays and were included in the confirmation profile.

A procedure has been developed for the determination of antidepressants and some of their metabolites in oral fluid using solid-phase extraction and LC–MS–MS analysis. The method was fully validated and was applied to research specimens.

Materials and Methods

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 1 mL of oral fluid ($\pm 10\%$) has been collected. This volume was determined using fifty subjects who were asked to use the Quantisal oral fluid collection device following the collection procedure instructions. The amount of oral fluid collected after activation of the volume adequacy indicator was determined gravimetrically by subtracting the weight of the individual device prior to collec-

tion from the weight post-collection. The mean volume for all 50 subjects was 0.993 mL with a standard deviation of 0.029 mL. Upon calculating the mean volume ± 3 times the standard deviation, the range obtained is 0.907 to 1.079. Hence, the Quantisal oral fluid device was determined to collect 1 mL $\pm 10\%$ of neat oral fluid.

The pad is then placed into transport buffer (3 mL), allowing a total 4-mL specimen volume available for analysis (3 mL buffer + 1 mL oral fluid). This is specifically advantageous in cases when 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. The buffer ensures stability of the drugs in the collection system during transportation to a testing facility.

Standards and reagents

A Tricyclic Antidepressants Direct ELISA kit was the primary assay used for screening the oral fluid. Direct ELISA kits for fluoxetine and sertraline were also used for screening those specific drugs. All ELISA kits were obtained from Immunalysis. Two deuterated internal standards, one to represent the second generation of antidepressants, fluoxetine- d_6 , and one to represent the older generation of drugs, nortriptyline- d_3 , were obtained from Cerilliant (Round Rock, TX). The unlabelled drugs: cyclobenzaprine, imipramine, dothiepin, doxepin, fluoxetine, sertraline, trimipramine, protriptyline, amitriptyline, clomipramine, nortriptyline, chlorpromazine, desmethyldoxepin, desipramine, desmethyltrimipramine, and norchlorimipramine were also purchased from Cerilliant. Solid-phase extraction columns (Clin II, 691-0353T) were obtained from SPEWare (San Pedro, CA). All solvents were HPLC-grade, and all chemicals were ACS-grade (Spectrum Chemicals, Gardena, CA).

Calibrators

For the chromatographic calibration standards, a working solution for the deuterated internal standards and unlabelled drug standards were prepared in methanol. All working solutions were stored at -20°C . For each batch, seven 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 negative human oral fluid samples, was prepared as follows: 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 oral fluid primarily because of the amount required in order to carry out all the experiments. The viscosity and pH of the synthetic oral fluid mimic that of the authentic matrix. Further, the effect of real oral fluid on the drugs compared to the effect of synthetic material is minimized during the 1+3 dilution with transportation buffer. Drug concentrations of 5, 10, 25, 50, 100, 250, and 500 ng/mL of neat oral fluid equivalents were prepared.

Screening assay

ELISA technology is based upon the competitive binding to an 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 25 ng/mL for nortriptyline, which is representative of the antidepressant class, using a broad spectrum assay that showed cross-reactivity with a range of drugs of similar structure. 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 μ L. 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. Characterization of the immunoassay in terms of cross-reactivity to the various drugs was determined by the manufacturer at equivalent concentrations to 25 ng/mL of nortriptyline (Table I).

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 (50 μ L of 250 ng/mL solution). 0.1 M potassium phosphate buffer (pH 6.0; 1 mL) was added to each calibrator, control, or oral fluid specimen. Solid-phase mixed-mode extraction columns (cation exchange: hydrophobic) were placed into a positive pressure manifold. Each column was conditioned with 2 mL methanol 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 2 mL deionized water followed by 1 mL 0.1 M hydrochloric acid and 1 mL methanol. The columns were allowed to dry under nitrogen pressure (5 min). The drugs were finally eluted using

3 mL freshly prepared methylene chloride/methanol/ammonium hydroxide (78:20:2, v/v/v). The extracts were evaporated to dryness under nitrogen and reconstituted in 40 μ L methanol.

LC-MS-MS

A 1200 series LC pump coupled to a 6410 triple-quadrupole MS operating in positive ESI mode was used for analysis (Agilent, Santa Clara, CA). The LC column was an Agilent Zorbax Eclipse XDB C18 (4.6 \times 50mm \times 1.8 μ m). The column temperature was held at 45°C, and the injection volume was 5 μ L. The mobile phase consisted of 0.2% acetic acid (Solvent A) and methanol (Solvent B). Initially, the mobile phase composition was 70% A/30% B at a flow rate of 1 mL/min. Over 8 mins, the percentage of methanol was increased to 70%; and equilibration time was 5 min. The gas temperature was 350°C, the gas flow was 13 L/min, and the nebulizer pressure was 60 psi. Nitrogen was used as the collision gas, and the capillary voltage was 4000 V. Two transitions were selected and optimized for each drug using flow injection analysis. The dwell time for all transitions was 25 ms, and optimal fragmentor and collision energy voltages were determined. Many procedures using MS-MS monitor only one transition for identification and quantitation of drugs in biological matrices; however, the monitoring of a second transition, allowing the ratio between the abundance of the primary and secondary transitions to be calculated, assures greater confidence in the final result. In this procedure, the ratio of the intensity of qualifier transition to the quantifier transition for each drug was determined at approximately the mid-point of the calibration range: 50 ng/mL. Once the ratio is determined from known drug standards, unknown peaks in the specimens are required to produce a transition ratio within \pm 20% of that determined from the drug standard in order for identification to be established.

Method validation

Linearity and sensitivity

Calibration using deuterated internal standards was calculated using linear regression analysis over a concentration range of 5–500 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 not forced through the origin. 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. Because all specimens were quantified, the limit of detection was not determined.

Drug recovery from the pad, accuracy, and precision

One of the main issues associated with oral fluid analysis is the recovery of drug from a collection pad. Therefore, the efficiency of antidepressant extraction from the collection device

Table I. Cross-Reactivity of Various Antidepressants in the Immunalysis ELISA Assay

Drug	Approximate Equivalent to Nortriptyline at 25 ng/mL	Cross-Reactivity (%)
Amitriptyline	12.5	200
Chlorpromazine	60	40
Clomipramine	60	40
Cyclobenzaprine	30	83
Desipramine	12.5	200
Desmethyldoxepin	160	15
Dothiepin	100	25
Doxepin	160	15
Fluoxetine	> 10,000	0
Imipramine	12.5	200
N-Desmethyltrimipramine	50	50
Norclomipramine	125	20
Nortriptyline	25	100
Protriptyline	100	25
Sertraline	> 10,000	0
Trimipramine	50	50

was determined. Synthetic oral fluid was fortified with drugs at a concentration of 40 ng/mL. A collection pad was placed into the fluid until the volume adequacy indicator turned blue, which means that 1 mL ($\pm 10\%$) of oral fluid had been absorbed. The pads were then placed into 3 mL Quantisal buffer, 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 1-mL aliquot of the specimen was analyzed. The procedure was repeated six times.

The accuracy of the procedure was determined over six replicates at 40 ng/mL. Accuracy was calculated as follows: (mean measured concentration – fortified concentration)/the fortified concentration $\times 100\%$.

Inter- and intraday precision of the assays was determined at a concentration of 40 ng/mL. Intraday data were obtained from six analyses performed on one day; interday data were obtained by analyzing six specimens each day for five days ($n = 30$).

Selectivity

Ion suppression. Oral fluid specimens were obtained from drug-free volunteers ($n = 6$), extracted, and analyzed according to the described procedures in order to assess interference from extraction or matrix or potential ion suppression. Ion suppression is more prevalent in the operational mode of ESI rather than atmospheric pressure chemical ionization. The 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. Published protocols from Matuszewski (14) and Chambers (15) suggest methods of post-extraction spiking of drug-free matrices and assessment of matrix effects and process efficiency. In order to perform experiments according to these protocols, a non-extracted 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).

The recovery of the antidepressants from the oral fluid ma-

Table II. Multiple Reaction Monitoring Transitions and Optimized Fragmentation Voltages for Antidepressant Drugs in Oral Fluid

Compound	Transition*	Fragment Voltage (V)	Collision Energy (eV)
Nortriptyline-d ₃	267 > 233	100	10
Doxepin	280 > 235 280 > 107	120 120	10 20
Desmethyldoxepin	266 > 235 266 > 107	100 100	10 20
Dothiepin	296 > 251 296 > 225	120 120	15 15
Cyclobenzaprine	276 > 216 276 > 231	110 110	10 20
Imipramine	281 > 86 281 > 58	100 100	10 30
Desipramine	267 > 72 267 > 44	110 110	15 30
Protriptyline	264 > 191 264 > 155	110 110	30 20
Nortriptyline	264 > 233 264 > 91	100 100	10 25
Amitriptyline	278 > 233 278 > 91	100 100	10 20
N-Desmethyltrimipramine	281 > 193 281 > 86	110 110	30 10
Trimipramine	295 > 100 295 > 58	110 110	10 30
Fluoxetine-d ₆	316 > 154	100	2
Fluoxetine	310 > 148 310 > 43	100 100	1 10
Chlorpromazine	319 > 86 319 > 58	110 110	25 30
Sertraline	306 > 275 306 > 129	35 35	10 20
Clomipramine	315 > 86 315 > 58	110 110	15 35
Norclomipramine	301 > 242 301 > 72	110 110	20 15

* Underlined transition used as quantifiers.

trix was determined by first assessing the response of the extracted samples ($n = 6$) at a concentration of 50 ng/mL (response of extracted specimens, RES). Next, oral fluid was extracted and drug was added post-extraction at a concentration of 50 ng/mL ($n = 6$) (response of post-extracted specimens, RPES). The percentage recovery was then calculated from the equation $(RES/RPES) \times 100$.

The percentage reduction/improvement in response to matrix effects (ion suppression/ion enhancement) was determined by assessing the peak area response of a non-extracted neat drug standard ($n = 6$) at a concentration of 50 ng/mL (response of non-extracted specimens, RNES). The non-extracted solution was analyzed in the same reconstitution solvent as the extracted specimens. The % matrix effect was then calculated using the equation $[(RPES/RNES) - 1] \times 100$. A negative result indicated ion suppression, and a positive result indicated ion enhancement of the signal. The overall efficiency of the process was calculated as $(RES/RNES) \times 100$.

Van Eeckhaut et al. (16) recently published an extremely useful review article of validated LC-MS-MS procedures where matrix effects had been discussed; however, there seemed to be no overall consensus on how this should be carried out. Reduction or elimination of matrix effects was best achieved by utility of deuterated internal standards wherever possible, extensive matrix clean-up before injection, and by optimized chromatographic and mass spectral conditions.

Exogenous interference. In order to assess potential problems arising from exogenous sources, 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 at a concentration of 10,000 ng/mL: cocaine, benzoylecgonine, cocaethylene, norcocaine, morphine, 6-acetylmorphine, codeine, hydrocodone, hydromorphone, oxycodone, oxymorphone, tramadol, fentanyl,

tetrahydrocannabinol, 11-nor- Δ^9 -carboxytetrahydrocannabinol, amphetamine, methamphetamine, methylenedioxymethamphetamine (MDMA), methylenedioxyamphetamine (MDA), methylenedioxyethylamphetamine (MDEA), carisoprodol, methadone, diazepam, nordiazepam, oxazepam, alprazolam, chlordiazepoxide, bromazepam, temazepam, lorazepam, flurazepam, nitrazepam, triazolam, secobarbital, pentobarbital,

Table III. Mean Linearity, Correlation (r^2), and Established Range for Quantifying/Qualifying Transition Ratio at 50 ng/mL ($n = 5$)

Analyte	Equation	Correlation (r^2)	Range for Qualifying Transition to Meet Criterion for Positivity ($\pm 20\%$)
Amitriptyline	$y = 0.0166x + 0.0341$	0.9962	62.9–94.4%
Chlorpromazine	$y = 0.5429x + 0.3353$	0.9974	74.6–111%
Clomipramine	$y = 0.8128x + 0.7030$	0.9978	47.2–70.8%
Cyclobenzaprine	$y = 0.2426x + 0.2128$	0.9962	47.3–70.9%
Desipramine	$y = 0.5310x + 0.7395$	0.9952	17.4–26.2%
Desmethyldoxepin	$y = 0.3025x + 0.4304$	0.9972	37.7–56.6%
Dothiepin	$y = 0.1037x + 0.1096$	0.9960	61.6–92.4%
Doxepin	$y = 0.1379x + 0.1855$	0.9972	27.8–41.8%
Fluoxetine	$y = 0.1609x + 0.0976$	0.9984	13.0–19.5%
Imipramine	$y = 0.9953x + 1.6817$	0.9948	20.2–30.3%
N-Desmethyltrimipramine	$y = 1.0719x + 1.1573$	0.9966	4.82–7.24%
Norclomipramine	$y = 0.4757x + 0.5369$	0.9964	6.34–9.52%
Nortriptyline	$y = 0.0217x + 0.0195$	0.9988	59.3–89.0%
Protriptyline	$y = 0.0299x + 0.0385$	0.9970	61.6–92.4%
Sertraline	$y = 0.138x + 0.1922$	0.9928	17.2–25.8%
Trimipramine	$y = 0.547x + 0.3629$	0.9974	63.7–95.6%

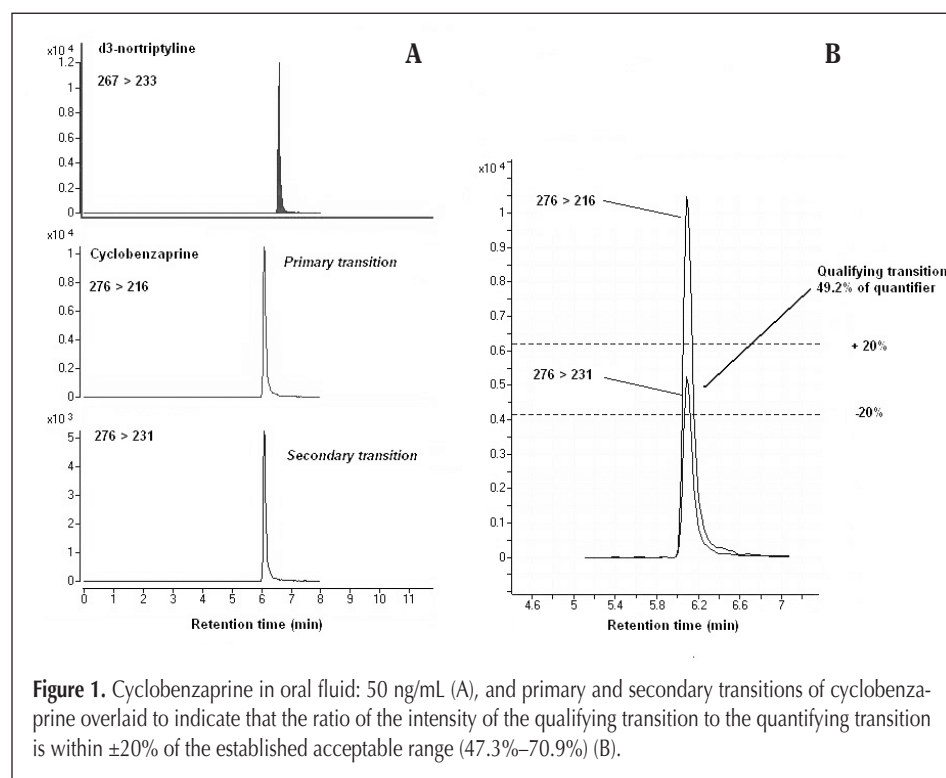


Figure 1. Cyclobenzaprine in oral fluid: 50 ng/mL (A), and primary and secondary transitions of cyclobenzaprine overlaid to indicate that the ratio of the intensity of the qualifying transition to the quantifying transition is within $\pm 20\%$ of the established acceptable range (47.3%–70.9%) (B).

butalbital, and phenobarbital.

Stability

The stability of the drug extracts at a concentration of 25 ng/mL was determined by allowing the autosampler vials to remain in the autosampler at 7°C for 48 h after which time they were re-analyzed. The responses were compared to those achieved on the first day of analysis.

Application of the procedure

As part of various ongoing studies, our laboratory receives oral fluid specimens for research purposes. The oral fluid specimens were screened using ELISA for the detection of tricyclic antidepressants as well as individual ELISA assays for sertraline and fluoxetine.

Results

Method validation

The LC-MS-MS procedure developed for the antidepressants was validated according to accepted protocols (17). Two internal standards were selected for the method: one represented the older generation of tricyclic antidepressants (nortriptyline- d_3) and one represented the second generation of antidepressant SSRIs (fluoxetine- d_6). Deuterated nortriptyline was used for the quantitation of nortriptyline, amitriptyline, cyclobenzaprine, desipramine, desmethyldoxepin, doxepin, dothiepin, imipramine, protriptyline, desmethyltrimipramine, and trimipramine. Deuterated fluoxetine was used for the quantitation of fluoxetine, sertraline, chlorpromazine, nor-

clomipramine, and clomipramine. For each drug, two transitions were monitored from the precursor ion (M+1). The primary and secondary transitions, voltages required for optimal fragmentation, and collision are shown in Table II.

Linearity and sensitivity

The LLOQ 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 range from 5 to 500 ng/mL of oral fluid. The calibration fit used was linear with a $1/x$ weighting. The mean correlation of the calibration curves; average linearity, where x = concentration of drug and the relative response, where y = peak area response of the drug/peak area response of the internal standard; and the ratio of the intensity of the qualifying transitions to the intensity of the quantifying transitions, along with an acceptable range for the determination of positivity, established at a concentration of 50 ng/mL are shown in Table III.

The most intense transition produced by fragmentation of the drug is monitored as the primary transition (e.g., cyclobenzaprine m/z 276 > 216) and is used for quantitation of the target compound. The secondary fragmentation uses the same precursor ion (M+1) and fragments to m/z 231 (Figure 1A). At a concentration of 50 ng/mL, the acceptable ratio of the qualifying transition was determined to be between 47.3 and 70.9% (Table III; Figure 1B).

Recovery, accuracy, and precision

The precision of the assay interday and intraday is shown in Table IV. Fluoxetine showed the highest variability both within and between days (6.2% and 8.2%, respectively). The accuracy of the assay for all drugs was more than 96%. The recovery of the various antidepressants from the collection pad using the

Quantisal device was determined and is also shown in Table IV. Although most of the drugs showed high recovery (> 70%), a few did not produce high yield even after overnight incubation in the transportation buffer. Specifically, sertraline, norclomipramine, and chlorpromazine were all recovered at less than 60%.

Selectivity

Ion suppression. The oral fluid is diluted during collection, deuterated internal standards are added, and specific solid phase procedures are employed. Overall these steps in the procedure contributed to minimal ion suppression: matrix effects of less than 25% for all drugs and process efficiencies higher than 70% in all cases (Table V).

Interference. Oral fluid specimens collected from drug-free in-

Table IV. Recovery of Antidepressants from Oral Fluid Collection Pad* Accuracy and Precision of the Assay

Drug	Recovery ($n = 6$) CV (%)	Accuracy (%)	Interday Precision ($n = 30$) (%)	Intraday Precision ($n = 6$) (%)
Amitriptyline	74.0 (2.8)	104	6.0	2.4
Chlorpromazine	59.4 (7.9)	100	3.7	1.8
Clomipramine	63.2 (5.1)	102	3.5	1.5
Cyclobenzaprine	78.3 (7.0)	103	3.5	4.2
Desipramine	78.7 (7.1)	105	7.2	2.6
Desmethyldoxepin	84.1 (8.4)	102	5.6	5.0
Dothiepin	80.3 (5.8)	96.2	5.6	5.8
Doxepin	87.3 (8.9)	99.1	5.8	2.0
Fluoxetine	76.4 (15.4)	101	8.2	6.2
Imipramine	87.4 (8.4)	102	2.9	1.8
N-Desmethyltrimipramine	73.6 (6.8)	101	4.4	3.4
Norclomipramine	51.5 (7.7)	103	3.6	2.5
Nortriptyline	68.2 (4.6)	100	4.0	1.8
Protriptyline	69.8 (5.6)	96.2	3.5	2.1
Sertraline	51.4 (6.5)	99.5	4.7	4.6
Trimipramine	81.6 (8.2)	99.1	4.2	2.0

* At a concentration of 40 ng/mL.

dividuals ($n = 6$) showed no interference with any of the assays, which was not unexpected because 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 Experimental Section. No chromatographic interference was observed in the channels of these transitions.

Stability

Finally, the stability of the drugs in the extracts was assessed. The extracts were stable for at least two days when kept inside the autosampler, which was maintained at 7°C. There was less than a 5% difference in the quantitation of the extracts after 48 h.

Discussion

Immunoassay screening

When a high number of specimens are to be analyzed, an immunochemical screening procedure can be efficient. However, a screening technique for a broad range of analytes may cause specimens not to be reported if individual compounds are not included in the confirmation profile. In October 2008, the National Safety Council (NSC) issued the following statement: "The National Safety Council and its Committee on Alcohol and Other Drugs strongly recommends that all presumptive positive drug screen results in samples obtained following transportation accidents be confirmed by an alternate analytical method prior to issuing a report. The practice of reporting

presumptive positive results in these cases should be abolished...." (<http://www.nsc.org>).

Because the ELISA system described was utilized in part for traffic safety projects, the ability to confirm cross-reacting species is necessary. Van Hoey (18) reported on the interference of cyclobenzaprine in tricyclic antidepressant screening assays and recommended it be included in any confirmation testing. Caravati et al. showed potential cross-reactivity of the anti-psychotic drug quetiapine in the Abbott TDx TCA assay (19). In this research, cyclobenzaprine was included in the confirmation profile because of its inhibition of the ELISA assay, but because quetiapine did not show significant cross-reactivity in the system used for this screening (< 0.25%), it was not included in the LC-MS-MS assay. Although chlorpromazine is not frequently used in the treatment of depression, it was included in the confirmation profile because it is primarily used as an anti-psychotic medication and has considerable cross-reactivity with the ELISA.

Drug recovery from the collection device

The recovery of drugs from a collection device is an essential component in the validation and application of oral fluid testing. The efficient recovery of various compounds from the Quantisal device has been extensively reported. Groschl et al. recently reported good recovery of glucocorticoids and peptides using the Quantisal, (20) and various authors have shown excellent recovery of cocaine, opiates, amphetamines, THC (21), tramadol, oxycodone, meperidine (22), and benzodiazepines (23) from the collection system. In this study, the recovery of sertraline, chlorpromazine, and norclomipramine were all less than 60%. Clomipramine, protriptyline, and nortriptyline showed less than 70% recovery, which indicates that not all drugs are recovered to an equal extent from a collection device, and studies involving oral fluid devices must consider the efficiency of drug recovery at relevant drug concentration.

Authentic specimens

The immunoassay described here is currently used in research projects for specimens received into our facility and was employed in the National Roadside Survey in 2007. The expansion of the LC-MS-MS profile described in this paper allows the confirmation of more antidepressant drugs. There is only one paper in the literature showing the comparison of antidepressants in both oral fluid and plasma (13). In 2008, de Castro et al. demonstrated the analysis of nine antidepressants and some metabolites using LC-MS-MS. They reported oral fluid concentrations from subjects receiving

Table V. Ion Suppression for Antidepressant Drugs Using LC-MS-MS: Recovery, Matrix Effect, and Process Efficiency

Drug (50 ng/mL)	Recovery (%) (R_{ES}/R_{PES}) × 100*	Matrix Effect (%) [(R_{PES}/R_{NES}) - 1] × 100*	Process Efficiency (%) (R_{ES}/R_{NES}) × 100*
Amitriptyline	91.6	-15.1	78
Chlorpromazine	88.0	-15.6	74
Clomipramine	93.8	-17.7	77
Cyclobenzaprine	95.2	-15.4	81
Desipramine	92.8	-13.5	80
Desmethyldoxepin	97.0	-22.3	75
Dothiepin	89.2	-18.0	73
Doxepin	92.6	-13.7	80
Fluoxetine	93.0	-19.2	75
Imipramine	93.6	-15.9	79
N-Desmethyltrimipramine	95.6	-14.4	82
Norclomipramine	93.0	-16.4	78
Nortriptyline	93.9	-10.7	84
Protriptyline	94.5	-11.8	83
Sertraline	93.7	-17.6	77
Trimipramine	93.9	-12.8	82

* R_{ES} = response of extracted specimens ($n = 6$); R_{PES} = response of post-extracted specimens ($n = 6$); and R_{NES} = response of non-extracted drug standards ($n = 6$).

150 mg daily doses of venlafaxine to be in excess of 200 ng/mL. Daily dosing of 50 mg of amitriptyline produced both amitriptyline and nortriptyline concentrations in excess of 20 ng/mL, and fluoxetine was detected at concentrations over 20 ng/mL following 20 mg daily dosing. All these drugs had saliva/plasma (S:P) ratios greater than 1. However, other antidepressants such as clomipramine and sertraline appeared to have a very low S:P ratio (< 0.2). The drugs were measured at concentrations of less than 5 ng/mL in oral fluid following routine dosing of 75 mg clomipramine and less than 2 ng/mL in oral fluid following dosing of 100 mg sertraline per day.

Limitations of the study

There are two important limitations associated with this study. Firstly, although the utility of a broad spectrum ELISA can be helpful for general screening, if a specific drug application is required and the reported cross-reactivity is low, then calibration of the immunoassay with the lower cross-reacting drug may be a useful option to improve the likelihood of detection.

Secondly, because the recovery of some drugs from the Quantisal device was low, particularly norclomipramine and chlorpromazine, a lowered cut-off concentration may be more appropriate for oral fluid screening, rather than the recommended 25 ng/mL in order to compensate for less efficient drug recovery. Using a lowered cut-off not only aids with detection of therapeutic dosing for some of the drugs but may also help to identify those with low cross-reactivity in the immunoassay. Further, low drug removal from the collection pad will also impact the limits of quantitation necessary in the confirmatory test, so techniques with improved sensitivity are increasingly useful in oral fluid analysis.

Conclusions

The determination of a wide range of antidepressants in oral fluid is described using both screening and confirmation technology. The immunoassay screening process was selected based on the target analyte and degree of cross-reactivity with drugs relevant to the specific application. The LC-MS-MS procedure is reproducible, robust, and precise. The assay includes the monitoring of a primary (quantifying) transition and a secondary (qualifying) transition, the ratio of which is required to be within 20% of that produced from a known calibration standard for definitive drug identification.

References

1. A. Brunnauer and G. Laux. Antidepressants and driving ability. *Psychiatr. Prax.* **30(Suppl 2)**: S102–105 (2003) (in German).
2. A. Brunnauer, G. Laux, E. Geiger, M. Sovka, and H.J. Moller. Antidepressants and driving ability: results from a clinical study. *J. Clin. Psychiatr.* **67(11)**: 1776–1781 (2006).
3. M. Wingen, J.G. Ramaekers, and J.A. Schmitt. Driving impairment in depressed patients receiving long-term antidepressant treatment. *Psychopharmacol.* **188(1)**: 84–91 (2006).
4. M.F. Hilton, Z. Staddon, J. Sheridan, and H.A. Whiteford. The impact of mental health symptoms on heavy goods vehicle drivers' performance. *Accid. Anal. Prev.* **41(3)**: 453–461 (2009).
5. T.P. Rohrig and L.J. Goodson. A sertraline-intoxicated driver. *J. Anal. Toxicol.* **28(8)**: 689–691 (2004).
6. J. Shen, H.J. Moller, X. Wang, S.A. Chung, G.K. Shapiro, X. Li, and C.M. Shapiro. Mirtazapine, a sedating antidepressant, and improved driving safety in patients with major depressive disorder: a prospective, randomized trial of 28 patients. *J. Clin. Psychiatr.* **70(3)**: 370–377 (2009).
7. K. Iwamoto, M. Takahashi, Y. Nakamura, R. Ishihara, Y. Uchiyama, K. Ebe, A. Nosa, Y. Nosa, K. Yoshida, T. Iidaka, and N. Ozaki. The effects of acute treatment with paroxetine, amitriptyline, and placebo on driving performance and cognitive function in healthy Japanese subjects: a double-blind crossover trial. *Hum. Psychopharmacol.* **23(5)**: 399–407 (2008).
8. K. Iwamoto, Y. Kawamura, M. Takahashi, Y. Uchiyama, K. Ebe, K. Yoshida, T. Iidaka, Y. Noda, and N. Ozaki. Plasma amitriptyline level after acute administration, and driving performance in healthy volunteers. *Psychiatr. Clin. Neurosci.* **62(5)**: 610–616 (2008).
9. G.F. Kauert, S. Iwersen-Bergmann, and S. Toennes. Assay of Δ^9 -tetrahydrocannabinol (THC) in oral fluid-evaluation of the Ora-Sure oral specimen collection device. *J. Anal. Toxicol.* **30(4)**: 274–277 (2006).
10. B.N. Patel, N. Sharma, M. Sanyal, and P.S. Shrivastav. Liquid chromatography tandem mass spectrometry assay for the simultaneous determination of venlafaxine and O-desmethylenlafaxine in human plasma and its application to a bioequivalence study. *J. Pharm. Biomed. Anal.* **47(3)**: 603–611 (2008).
11. B.N. Patel, N. Sharma, M. Sanyal, and P.S. Shrivastav. Analysis of second-generation antidepressant drug, sertraline and its active metabolite, N-desmethyl sertraline in human plasma by a sensitive and selective liquid chromatography–tandem mass spectrometry method. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **877(3)**: 221–229 (2009).
12. M. Concheiro, A. De Castro, O. Quintela, A. Cruz, and M. Lopez-Rivadulla. Determination of illicit and medicinal drugs and their metabolites in oral fluid and preserved oral fluid by liquid chromatography–tandem mass spectrometry. *Anal. Biochem. Chem.* **391(6)**: 2329–2338 (2008).
13. A. De Castro, M. Concheiro, O. Quintela, A. Cruz, and M. López-Rivadulla. LC-MS/MS method for the determination of nine antidepressants and some of their main metabolites in oral fluid and plasma. Study of correlation between venlafaxine concentrations in both matrices. *J. Pharm. Biomed. Anal.* **48(1)**: 183–193 (2008).
14. B.K. Matuszewski, M.L. Constanzer, and C.M. Chavez-Eng. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Anal. Chem.* **75**: 3019–3030 (2003).
15. E. Chambers, D.M. Wagrowski-Diehl, Z. Lu, and J.R. Mazzeo. Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. *J. Chromatogr. B* **852**: 22–34 (2007).
16. A. Van Eckhaut, K. Lanckmans, S. Sarre, I. Smolders, and Y. Michotte. Validation of bioanalytical LC-MS/MS assays: Evaluation of matrix effects. *J. Chromatogr. B* **877(23)**: 2198–2207 (2009).
17. F.T. Peters, O.H. Drummer, and F. Musshoff. Validation of new methods. *Forensic Sci. Int.* **165**: 216–224 (2007).
18. N.M. Van Hoey. Effect of cyclobenzaprine on tricyclic antidepressant assays. *Ann. Pharmacother.* **39(7–8)**: 1314–1317 (2005).
19. E.M. Caravati, J.M. Juenke, B.I. Crouch, and K.T. Anderson. Quetiapine cross-reactivity with plasma tricyclic antidepressant immunoassays. *Ann. Pharmacother.* **39(9)**: 1446–1449 (2005).
20. M. Groschl, H. Kohler, H.G. Topf, T. Rupprecht, and M. Rauh. Evaluation of saliva collection devices for the analysis of steroids, peptides and therapeutic drugs. *J. Pharm. Biomed. Anal.* **47**: 478–486 (2008).
21. O. Quintela, D.J. Crouch, and D.M. Andrenyak. Recovery of drugs of abuse from the Immunalysis Quantisal™ oral fluid col-

- lection device. *J. Anal. Toxicol.* **30(8)**: 614–616 (2006).
22. C. Moore, S. Rana, and C. Coulter. Determination of meperidine, tramadol and oxycodone in human oral fluid using solid phase extraction and gas chromatography–mass spectrometry. *J. Chromatogr. B Biomed. Appl.* **850**: 370–375 (2007).
 23. C. Moore, C. Coulter, K. Crompton, and M. Zumwalt. Determination of benzodiazepines in oral fluid using LC/MS/MS. *J. Anal. Toxicol.* **31(9)**: 596–600 (2007).

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