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A study of matrix effects on an LC/MS/MS assay for olanzapine and desmethyl olanzapine

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Abstract

The purpose of this research project was to investigate potential matrix effects of anticoagulant and lipemia on the response of olanzapine, desmethyl olanzapine, olanzapine-D_3 and desmethyl olanzapine-D_8 in an LC/MS/MS assay. Blank human serum and sodium heparin, sodium citrate, and K_3EDTA plasma with various degrees of lipemia were fortified with olanzapine, desmethyl olanzapine, olanzapine-D_3 and desmethyl olanzapine-D_8. Six replicates of each sample were extracted using Waters Oasis® MCX cartridges and analyzed using electrospray LC/MS/MS. The analytes were separated on a Phenomenex LUNA phenyl hexyl, 2 mm × 50 mm, 5 μm, analytical column and a gradient rising from 2 to 85% mobile phase B. Mobile phase A consisted of acetonitrile–ammonium acetate (20 mM) (52:48 v/v) and mobile phase B was formic acid–acetonitrile (0.1:100 v/v). Ion suppression was investigated through post column infusion experiments. The degree of lipemia of each sample, indicated by turbidity, was ranked into categories from least to greatest and used for statistical analyses. The results from analysis of variance testing indicated that lipemia, anticoagulant and their interaction significantly influenced mass spectral matrix effects and extraction matrix effects. Differential behavior between the analytes and labeled internal standards contributed to variability. The most significant source of variability however, was ion suppression due to co-eluting matrix components.

Keywords: Olanzapine; LC/MS/MS; Matrix effects; Ion suppression; Lipemia; Anticoagulant

1. Introduction

Olanzapine (OLZ), a thienobenzodiazepine (Fig. 1), is administered as a treatment for schizophrenia as an atypical antipyschotic. The efficacy of OLZ, which is equal to or greater than conventional antipyschotic drugs, has been demonstrated for alleviating positive and negative symptoms of schizophrenia with a relatively low occurrence of extrapyramidal side effects [1]. OLZ has been shown to exhibit a high affinity for serotonin, dopamine, muscarinic and histamine receptors [2]. Among the many metabolites formed by Phase I and Phase II pathways, desmethyl olanzapine (DES) (Fig. 1), which is formed via CYP1A2, and olanzapine 10-*N*-glucuronide are the primary metabolites detectable in human plasma. The high degree of

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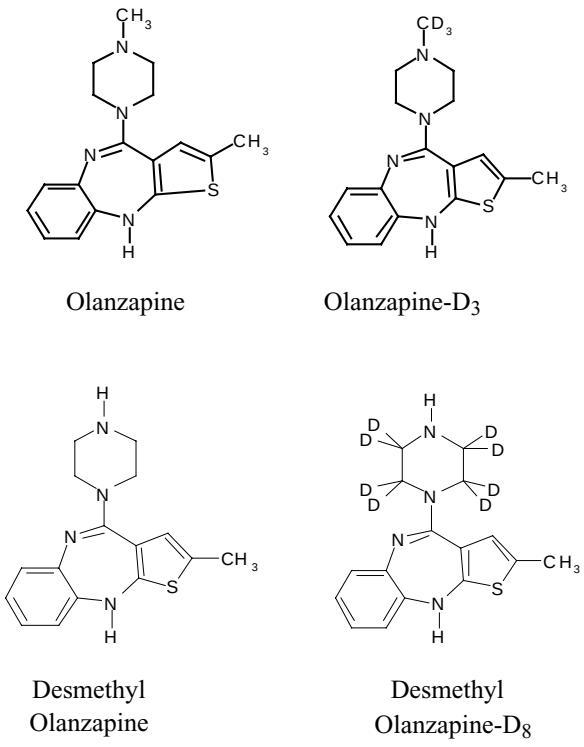

Fig. 1. Chemical structures of olanzapine, olanzapine-D_3, desmethyl olanzapine, desmethyl olanzapine-D_8.

interindividual and intraindividual variability of OLZ is well documented in the literature [3–5].

Many published methods for OLZ in human plasma or serum have been developed for therapeutic drug monitoring using LC-UV and LC-EC [6–10]. However, liquid chromatography/tandem mass spectroscopy (LC/MS/MS) methods have an important advantage over routine LC-UV and HPLC-EC methods because analysis time can often be reduced to under 5 min due to the selectivity of a mass spectrometer as a detector. However the published LC/MS/MS methods for olanzapine require large aliquot volumes and relatively long chromatographic analysis times of 7–9 min per injection which leaves room for improvement [6,11]. The method described here only requires a 0.20 ml sample and decreases the total run time to approximately 4 min.

The evaluation of selectivity is an important aspect of method development and validation. Interferences or matrix effects can compromise selectivity. Interference is a constant pre-determinate error and effects the response of the analyte to the same degree irrespective of analyte concentration [12]. Matrix effects result in a proportional error with respect to analyte concentration on the response of the analyte. Matrix effects are due to the occurrence of reactions between the analyte and some component of the matrix which affect the response of the analyte and are more difficult to evaluate than interferences. During the validation of a method, matrix effects can be investigated by the preparation of a number of samples in various matrices and quantitating the percent difference from nominal values and coefficient of variation from replicate extracts of these samples. A large percent difference from the nominal value and high coefficients of variation from these samples may indicate the possible occurrence of matrix effects and may warrant further investigation. According to the Food and Drug Administration's guidelines for bioanalytical method validations, matrix effects should be examined for LC/MS/MS methods [13].

One common matrix effect in the case of LC/MS or LC/MS/MS assays is ion suppression due to co-eluting matrix components which can affect the ionization efficiency of the analytes and therefore the response. This matrix effect can be investigated through post column infusion experiments or through the addition of the analyte to blank matrix extracts following extraction. If the response of the analyte is compared to an unextracted external standard solution, any difference from 100% recovery can be attributed to instrumental matrix effects. A combination of interferences and matrix effects can occur which may complicate the evaluation of selectivity.

Liquid chromatography coupled with tandem mass spectroscopy (LC/MS/MS) is one of the most powerful approaches in use today for the analysis of drug substances in biological matrices [14,15]. Two ionization mechanisms commonly used in atmospheric pressure ionization (API) are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). A problematic issue in ESI is that of ion suppression matrix effects. These matrix effects are the result of co-eluting components from the matrix that can cause a variable reduction in response. There are several possible mechanisms which occur during desolvation and ionization which could be responsible for a loss of analyte response [16]. In the positive mode,

if ionized analyte is transferred to the gas phase, gas phase proton transfer reactions may cause neutralization if another neutral species is present in the gas phase with a higher proton affinity than the analyte. Other ionic species, such as salts, in biological samples with high ionization efficiency or surface activity may compete with analytes during ion evaporation. High levels of nonvolatile substances may affect the transfer of ionized analyte into the gas phase by preventing the radius and surface charge of the droplets from reaching the levels necessary for ion emission [16,17]. It is hypothesized that APCI is associated with a smaller degree of ion suppression because analytes are already in the gas phase when molecular reactions occur and therefore the smaller degree of suppression observed with APCI is due to analyte precipitation [16]. APCI was not utilized for this project because the analyte response with APCI was five times less than with ESI. The lower limits of quantitation, 0.0500 and 0.100 ng/ml for OLZ and DES, respectively, could not be detected with APCI.

There are several techniques commonly employed to correct for ion suppression. Isotopically labeled internal standards are utilized because of their ability to mimic the extraction and instrumental behavior of the analyte. Ideally an isotopically labeled internal standard chromatographs at the same retention time as the analyte and therefore the amount of ion suppression due to co-eluting substances would effect both the analyte and labeled internal standard similarly. However, isotopically labeled internal standards can be difficult to synthesize and therefore may be expensive or unavailable. Another consideration is the case of multiple analytes in which a labeled internal standard would have to be available for each analyte. The use of post column internal standard addition has been reported by Choi et al. to correct for ion suppression in an LC/MS assay without requiring coelution of the analyte and internal standard but it does not correct for changes in analyte response caused by extraction or injection differences [18]. Improving the sample clean-up procedure to remove matrix components or diluting the sample, thereby decreasing the amount of matrix injected onto the column, can also reduce ion suppression [19,20]. However sample throughput may have to be sacrificed due to long or complicated extraction procedures. Two-dimensional liquid chromatography (2D-LC) has been used to investigate the effect of column overloading with respect to ion suppression which suggests that minimization of injection volume is essential to reduce ion suppression [21,22]. Another method to abate ion suppression is to chromatographically resolve the coeluting matrix effect from the analyte and/or internal standard, possibly at the expense of analysis time [23,24].

The effect of matrix components, such as anticoagulant and lipids, on analyte and internal standard response in this assay were examined in detail. The decision of which matrix to use for clinical samples can have significant implications. Lipemia is a result of high levels of lipids present in biological samples as lipoproteins [12,25–27]. Lipemic samples are turbid and the degree of "milkyness" is a function of very-low-density lipoproteins (VLDL) and chylomicron content. Any solid substance in a sample, whether it is a fibrin clot or insoluble lipid, has the potential to block the flow through a solid phase cartridge bed during extraction. Other problems related to instrumentation such as coeluting interferences and ion suppression effects are also important to investigate when utilizing LC/MS/MS. This project investigates the effect of anticoagulant, lipemia or an interaction between anticoagulant and lipemia on analyte and internal standard responses in this assay.

2. Experimental

2.1. Chemicals and reagent preparation

2.1.1. Chemicals and materials

Reagents and chemicals were of HPLC grade. Methanol and acetonitrile obtained from Burdick and Jackson (Muskegon, MI). Ammonium acetate, ≥99.99% was purchased from Sigma Chemical Co. (St. Louis, MO). Citric acid (monohydrate) and Dulbecco's phosphate buffered saline (10×) (PBS) were acquired from Aldrich Chemical Company (Milwaukee, WI). Ammonium hydroxide was purchased from J.T. Baker (Phillipsburg, NJ). Formic acid was bought from Mallinckrodt (Paris, Kentucky). Reagent water was produced by a ModuLab® ModuPure Plus reagent grade water system manufactured by Continental Water Systems Corporation (San Antonio, TX). PPD Discovery (Wilmington, NC) synthesized

olanzapine, olanzapine-D_3 (OLZ-D_3), desmethyl olanzapine and desmethyl olanzapine-D_8 (DES-D_8) standards. Individual donor lots of human plasma containing sodium heparin (Na heparin) and tripotassium ethylenediaminetetraacetic acid (K_3EDTA) were purchased from Biological Specialty Corporation (Colmer, PA). Sodium citrate (Na citrate) human plasma and human serum were obtained from Biochemed Pharmacologicals (Winchester, VA and Memphis, TN). Waters Oasis® MCX (30 mg, 1 ml) 96-well plates were utilized for extraction (Waters Corporation, Milford, MA).

2.1.2. Stock and working standard solutions

Stock solutions of OLZ, DES and DES-D_8 were prepared volumetrically at 100 μg/ml in methanol. An OLZ-D_3 stock was prepared at approximately 1 mg/ml in methanol. A 1000 ng/ml OLZ and 2000 ng/ml DES spiking solution was prepared in methanol to be used in the preparation of the specificity samples. A 1000 ng/ml OLZ-D_3 and DES-D_8 working internal standard solution was prepared in 50:50 methanol:water. A 1.5 ng/ml OLZ, 3.0 ng/ml DES, 125 ng/ml OLZ-D_3 and DES-D_8 external solution was prepared in 50:50 methanol:water for recovery and ion suppression experiments. The above solutions were stored at 5 °C or lower.

2.1.3. Preparation of specificity samples

Commercially available lots of K_3EDTA and sodium citrate human plasma, and serum from six individual donors and sodium heparin human plasma from nine individual donors were randomly selected. PBS was utilized as the control. Each blank was labeled as "SP X" where X is a number from 1 to 6 (9 for sodium heparin plasma). Middle level specificity samples, 1.5 ng/ml OLZ and 3.0 ng/ml DES, were prepared in each lot of plasma, serum or PBS. These specificity samples were labeled as "SPF X"s where X corresponded to a particular lot of blank. All of the blanks and samples were stored in polypropylene tubes at −20 °C.

2.1.4. Validation

A method was validated to analyze OLZ and DES with two isotopically labeled internal standards, OLZ-D_3 and DES-D_8 in sodium heparin human plasma. Calibration standards were prepared at 0.050, 0.100, 0.250, 0.500, 1.00, 2.50, 5.00, 10.0, 25.0 and 50.0 ng/ml OLZ and 0.100, 0.250, 0.500, 1.00, 2.50, 5.00, 10.0, 25.0, 50.0 and 100 ng/ml DES volumetrically. Quality controls were prepared at 0.0500, 0.100, 1.50 and 30.0 ng/ml OLZ and 0.100, 0.200, 3.00 and 60.0 ng/ml DES volumetrically. The calibration standards and quality controls were stored at −20 °C in polypropylene tubes until extracted.

2.1.5. Instrumentation

A Tomtec Quadra 96® Model 320 (Hamden, CT) was used to automate the solid phase extraction. The tomtec is designed to handle up to 96 samples simultaneously in a 96-well format. A SPE Dry-96 sampler concentrator from Jones Chromatography (Lakewood, CA) was used to evaporate the sample plate to dryness under nitrogen at a set temperature. The HPLC system consisted of a HP 1100 Series binary pump (Germany) and a Leap Technologies CTC LC PAL autosampler (Carrboro, NC) programmed to inject from 96-well plates. Phenomenex LUNA phenyl hexyl 2 mm × 150 mm, 5 μm, analytical columns (Torrance, CA) were used for separation in reverse phase. A PE Sciex API 3000 mass spectrometer (Foster City, CA) with a TurboIonSpray® source was used for analysis. Data acquisition was performed using a Macintosh workstation equipped with Macquan software (PE Sciex). A Varian Prospekt 9200 (Walnut Creek, CA) controlled the timing of the LC/MS/MS analysis. A Havard Apparatus Pump 11 syringe pump (Holliston, MA) was utilized for ion suppression experiments.

2.1.6. Extraction procedure for specificity experiments

Each lot of blank serum, plasma and PBS was extracted with the addition of the internal standards, termed "SP/IS", and without the addition of the internal standards, termed "SP". In addition six replicates of the middle level specificity samples, "SPF"s, were extracted with the following procedure.

A 0.20 ml aliquot of each sample was added to a well in a 2 ml, square well 96-well plate. Twenty-five microliters of working internal standard solution were added to each sample except for the blanks. Twenty-five microliters of methanol–water (50:50 v/v) were added to each blank. The samples were then acidified with 0.20 ml of citric acid (1 M), vortexed

and centrifuged. The Tomtec was used for further extraction steps.

An Oasis® MCX 96-well solid phase extraction plate was conditioned using 0.30 ml of citric acid (1 M) twice. Four hundred microliters of the sample in each well of the sample plate was then transferred to the extraction plate. A very low vacuum was applied to slowly load the cartridges with sample. The cartridges were washed twice with 0.35 ml of citric acid (1 M) and 0.35 ml of methanol. After each addition, a very slow vacuum was applied to slowly wash the cartridges. The cartridges were then dried by applying a full vacuum for approximately 1 min. Two 0.35 ml volumes of ammonium hydroxide–methanol (2.5:97.5 v/v) were used to elute the retained analytes and internal standards. The samples were evaporated to dryness under nitrogen at 40 °C and reconstituted with 0.20 ml of methanol–water (50:50 v/v). The extracts were vortexed and centrifuged for 1–2 min. Thirty microliters of the extracts were injected for LC/MS/MS analysis. The extracts were stored at 0–5 °C following analysis.

Some samples were fortified with internal standards only after evaporation was complete to be used for recovery comparisons. These samples were labeled "SP/IS Post". The samples were reconstituted with 25 μl of the 1000 ng/ml OLZ-D_3 and DES-D_8 working internal standard solution and 0.175 ml of methanol–water (50:50 v/v).

2.2. Instrumental conditions

2.2.1. HPLC conditions

An isocratic separation was validated for a flow rate of 0.4 ml/min consisting of 98% mobile phase A acetonitrile–ammonium acetate (20 mM) (52:48 v/v) and 2% mobile phase B formic acid–acetonitrile (0.1:100 v/v). Preliminary ion suppression testing indicated that the isocratic separation increased the potential for late eluting suppression affecting the results. Therefore specificity testing was investigated utilizing a gradient at a flow rate of 0.4 ml/min. The gradient employed a program in which the percent of mobile phase B increased from 2 to 85% during the first minute and immediately returned to 2% for the remainder of the analysis.

The approximate retention time for OLZ and OLZ-D_3 was 1:50 (min:s) For DES and DES-D_8 the approximate retention time was 1:35 (min:s).

2.2.2. Tandem mass spectrometer conditions

Electrospray ionization and analysis were accomplished using a TurboIonSpray® interface and a PE Sciex API 3000 mass spectrometer operated in the positive mode with multiple reaction monitoring. The *m/z* transitions and dwell times for OLZ, OLZ-D_3, DES and DES-D_8 were 312.9/256.0, 316.1/256.0, 299.1/256.0, 306.7/213.0, and 50, 40, 350 and 40 ms, respectively. The analysis was performed at 400 °C. The ionspray, orifice, ring and declustering potentials were set at 1500, 32, 263 and −350 V, respectively. Nitrogen was used as the curtain, nebulizer and collision gas. The scan time was set to 0.5 s and the pause time was 5 ms. Data was acquired for 3.9 min. The total run time for each injection controlled by the Varian Prospekt 9200 was 4 min and 10 s.

2.2.3. Ion suppression experiments

An external standard corresponding to the concentration of the middle level specificity extracts was infused post-column using a syringe pump at 10 μl/min into a tee and mixed with mobile phase. The run time was extended to approximately twice the normal run time to allow for the detection of late eluting suppression bands. Selected SP/IS samples were injected and the OLZ and DES channels were monitored for suppression. The internal standard channels were used to indicate the approximate retention times of the analytes since they co-eluted.

2.2.4. Categorical characterization of lipemia

Categories were visually created to describe various extents of lipemia (Table 1). Each blank was aliquoted into a 12 mm × 75 mm glass test tube and viewed against a black background.

Table 1
Lipemic characterization of each sample

	Lipemic category								
	Sample no.								
	1	2	3	4	5	6	7	8	9
K_3EDTA human plasma	1	2	4	3	3	1			
Sodium citrate human plasma	2	2	1	3	4	4			
Human serum	1	3	2	4	3	3			
Sodium heparin human plasma	3	1	1	1	1	1	4	4	4

Description of each category:

Category 1: Transparent when inspected from the side and top

Category 2: Transparent to slightly turbid when inspected from the side and not transparent when inspected from the top

Category 3: Barely transparent when inspected from the side and not transparent when inspected from the top

Category 4: Not transparent when inspected from the side or top

Some intrinsic bias was encountered in making visual observations concerning turbidity. It was difficult to distinguish between turbidity due to fibrin particles floating homogeneously throughout the sample and turbidity due to lipid content.

2.3. Statistical analyses

The data from the six replicates of each specificity sample was tested for outliers utilizing a range test at 95% significance [28]. Samples prepared in PBS were chosen as the control to determine matrix effects. The data was organized according to anticoagulant and lipemic category for testing purposes. Analysis of variance (ANOVA) was performed to elucidate any effect of lipemia, anticoagulant or an interaction between lipemia and anticoagulant on extraction recovery, matrix effects and extraction matrix effects using JMP 4.0 Professional software.

The data was initially tested for an interaction between lipemic category and anticoagulant. For the interaction testing, significance was determined by the conservative Bonferroni approach where $\alpha^* = \alpha/k$ [29]. A value of 0.05 was substituted for α for all calculations and k indicates the number of tests performed. If a significant interaction was found, slices of the means within a group were investigated to determine where the differences occurred. If no significant interaction was found, the ANOVA model was reduced to examine the effects of lipemia and anticoagulant separately. For these analyses, $\alpha = 0.05$ was utilized to determine significance. Tukey–Kramer honestly significant difference (HSD) tests were performed to indicate significant differences within the lipemic or anticoagulant categories [30].

3. Results and discussion

3.1. Results from previous validations

3.1.1. Calibration and quality control data

A linear, 1/concentration^2 weighted regression model was utilized to fit the calibration curve. The mean correlation coefficients ($n = 2$) were determined to be 0.9993 and 0.9987 for OLZ and DES, respectively. Intra-assay accuracy ($n = 6$) ranged from −3.44 to 9.42% for OLZ and 1.46 to 7.12% for DES. The intra-assay precision ($n = 6$) for OLZ and DES ranged from 2.09 to 5.01% and 0.806 to 6.01%, respectively. The inter-assay accuracy ($n = 2$) ranged from −4.70 to −6.47% for OLZ and 3.64 to 6.24% for DES. Inter-assay precision values ($n = 2$) were reported from 2.64 to 4.08% for OLZ and 1.44 to 5.42% for DES.

3.1.2. Variability and recovery from specificity experiments

The responses of the analytes and internal standards from specificity samples prepared using plasma in various lipemic categories were highly variable (Tables 2 and 3). This variability reflects extraction and ionization variability combined. High variability and low response occurred more frequently with severely lipemic samples. The overall coefficients of variation for each anticoagulant group were greater than 27%. These results indicate how variable the responses might be from a group of individual subjects with varying degrees of lipid content. The data illustrates the potential variability between individuals with different lipid concentrations or intraindividual variability from sampling at different time points, perhaps after a patient has ingested a meal high in fat. The analyte to internal standard ratios in Tables 2 and 3 were also inconsistent which indicates that the labeled internal standards were not effectively able to correct for the variability in analyte response. In order to obtain a clearer picture of problems with recovery, overall absolute, extraction and instrumental recoveries were calculated for each sample.

Overall absolute recovery represents the combined effects of extraction and ionization efficiency on the response of the analytes and internal standards. Overall absolute recovery was calculated by comparing the absolute area of each analyte and internal stan-

Table 2
Comparison of mean areas (%CV) and analyte to internal standard ratios (%CV) for olanzapine and olanzapine-D_3 for nine donor lots of sodium heparin human plasma ($n = 6$)

Sample name	Lipemic category	Olanzapine area	Olanzapine-D_3 area	Ratio
PBS SPF	N/A	243,124 (12.5)	933,895 (12.6)	0.260 (1.98)
SPF 1	3	315,244 (0.955)	900,697 (4.50)	0.356 (1.31)
SPF 2	1	194,209 (11.3)	582,517 (12.0)	0.334 (0.944)
SPF 3	1	402,425 (2.49)	1,019,465 (2.16)	0.395 (0.575)
SPF 4	1	359,478 (5.87)	974,080 (5.62)	0.369 (1.44)
SPF 5	1	305,347 (12.3)	806,462 (12.7)	0.376 (0.699)
SPF 6	1	340,399 (13.3)	867,541 (14.4)	0.393 (1.26)
SPF 7	4	9,020 (16.8)	516,810 (12.2)	0.017 (5.48)
SPF 8	4	6,400 (32.1)	385,191 (31.2)	0.017 (9.24)
SPF 9	4	5,014 (97.0)	127,571 (92.1)	0.038 (7.05)
Mean for sodium heparin human plasma		215,282 (77.1)	686,704 (44.0)	0.255 (68.4)

dard to that of an external standard representing 100% recovery (Fig. 2). The absolute recoveries for all of the specificity samples were below 70%. Considerable differences were observed between the recovery of the analytes and labeled internal standards for some samples, such as in SPF 7–9 prepared in sodium heparin plasma. This suggested that the internal standards had not effectively mimicked the behavior of the analytes either during extraction. Lipemia may not have been solely responsible for the internal standard tracking problem because differences occurred with plasma in the least as well as in the most severely lipemic categories. The severely lipemic samples (category 4) displayed high variability and a substantial loss of response which could not be attributed to poor chromatography.

In an attempt to elucidate the cause of the lower recovery for some samples, internal standard recovery was separated into absolute extraction and instrumental recovery. Absolute extraction recovery of the internal standards was calculated by comparing the absolute areas from blanks and samples fortified with internal standard, SP/IS and SPF samples, to blanks fortified with internal standard post extraction, SP/IS post samples (Fig. 3). Extraction recovery ranged from 40 to 90% for most of the samples although some samples such as sodium heparin SP/IS 2 and other samples in category 4 in the K_3EDTA, sodium citrate and

Table 3
Comparison of mean areas (%CV) and analyte to internal standard ratios (%CV) for desmethyl olanzapine and desmethyl olanzapine-D_8 for nine donor lots of sodium heparin human plasma ($n = 6$)

Sample name	Lipemic category	Desmethyl olanzapine area	Desmethyl olanzapine-D_8 area	Ratio
PBS SPF	N/A	78,179 (16.5)	2,600,710 (17.2)	0.0301 (1.31)
SPF 1	3	88,999 (7.36)	2,654,160 (3.18)	0.0323 (1.23)
SPF 2	1	42,577 (12.0)	1,881,255 (12.0)	0.0226 (1.23)
SPF 3	1	122,376 (3.60)	3,287,814 (8.80)	0.0385 (1.26)
SPF 4	1	94,833 (6.70)	2,925,683 (6.24)	0.0324 (1.96)
SPF 5	1	92,486 (9.74)	2,583,882 (8.71)	0.0358 (1.65)
SPF 6	1	103,927 (10.7)	2,656,779 (10.6)	0.0391 (1.55)
SPF 7	4	4,205 (19.8)	1,897,497 (22.5)	0.00223 (6.26)
SPF 8	4	2,107 (45.5)	960,679 (48.0)	0.00215 (4.98)
SPF 9	4	1,588 (106)	325,616 (115)	0.00540 (13.0)
Mean for sodium heparin human plasma		61,455 (79.5)	2,130,374 (45.4)	0.0234 (67.8)

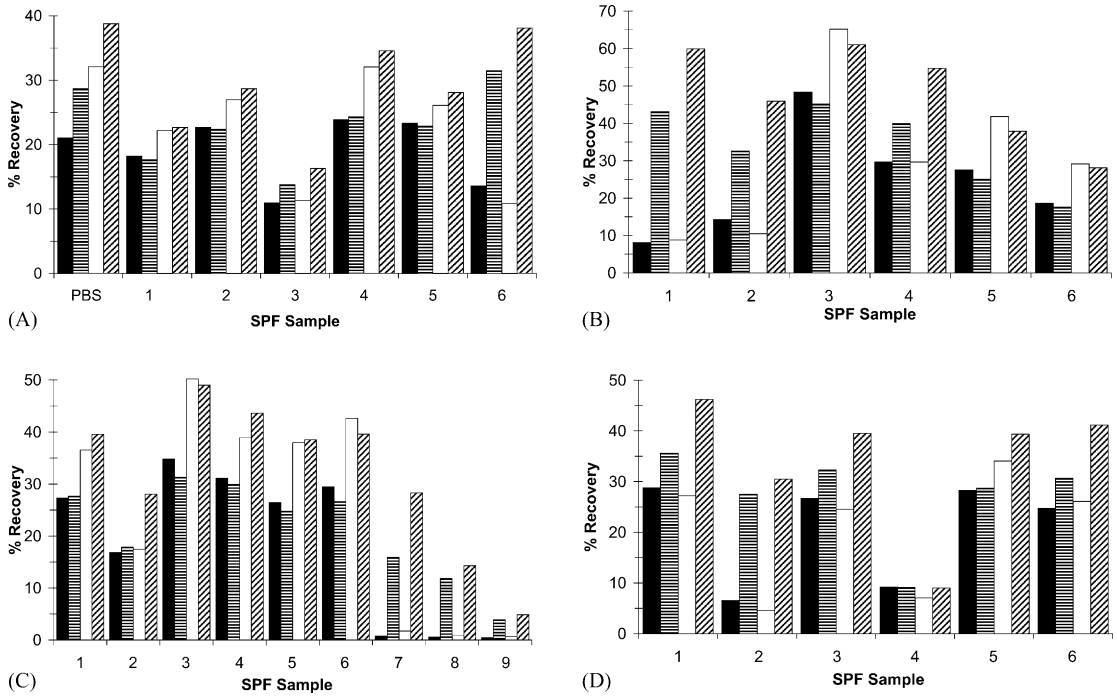

Fig. 2. Overall absolute recovery for specificity samples prepared in K_3EDTA human plasma (A), sodium citrate human plasma (B), sodium heparin human plasma (C) and human serum (D) (■, olanzapine; ▨, olanzapine-D_3; □, desmethyl olanzapine; ▤, desmethyl olanzapine-D_8).

serum group only demonstrated 20–30% recovery for both internal standards. This was evidence that lipemia was not the only factor affecting extraction recovery.

The MCX cartridges used in the extraction required very slow load, wash and elution steps and needed to be carefully controlled. Channeling or saturation could have resulted in poor recovery. Interference with the ionization of the analytes or internal standards could also have caused a decrease in recovery.

Absolute instrumental recovery was calculated by comparing the absolute areas from blanks fortified with internal standard post extraction, SP/IS post samples, to an external standard representing 100% recovery (Fig. 4). The instrumental recovery for OLZ-D_3 and DES-D_8 ranged from 34 to 54% and 55 to 80%, respectively. The recovery of some of the post spiked samples in lipemic category 4 such as sodium heparin SP/IS 7–9 were highly variable and significantly lower than the other lots in their respective groups. In addition to affecting extraction recovery and variability, lipemia also appeared to affect instrumental recovery, perhaps contributing to ion suppression.

3.2. Statistical analysis

Statistical analyses were performed to determine whether there were significant matrix effects due to lipemia or anticoagulant. A result of the random selection of donor lots was unequal sample sizes within each lipemic category and each anticoagulant. For example, sodium heparin human plasma did not contain any samples in lipemic category two. The effect of random selection was important to reproduce because when specificity samples are prepared in a validation, the lots are chosen randomly without any concern as to the degree of lipemia. Also the degree of lipemia in real patient samples cannot be predetermined. Random selection resulted in a zero cell for lipemic category 2 within the sodium heparin human plasma group and this caused some irregularities in the interaction analysis by JMP. The number of degrees of freedom for the interaction testing should be nine. However, since there was a zero cell, the number of degrees of freedom for the interaction analyses was determined to be eight. The practical effect of this inaccuracy is a

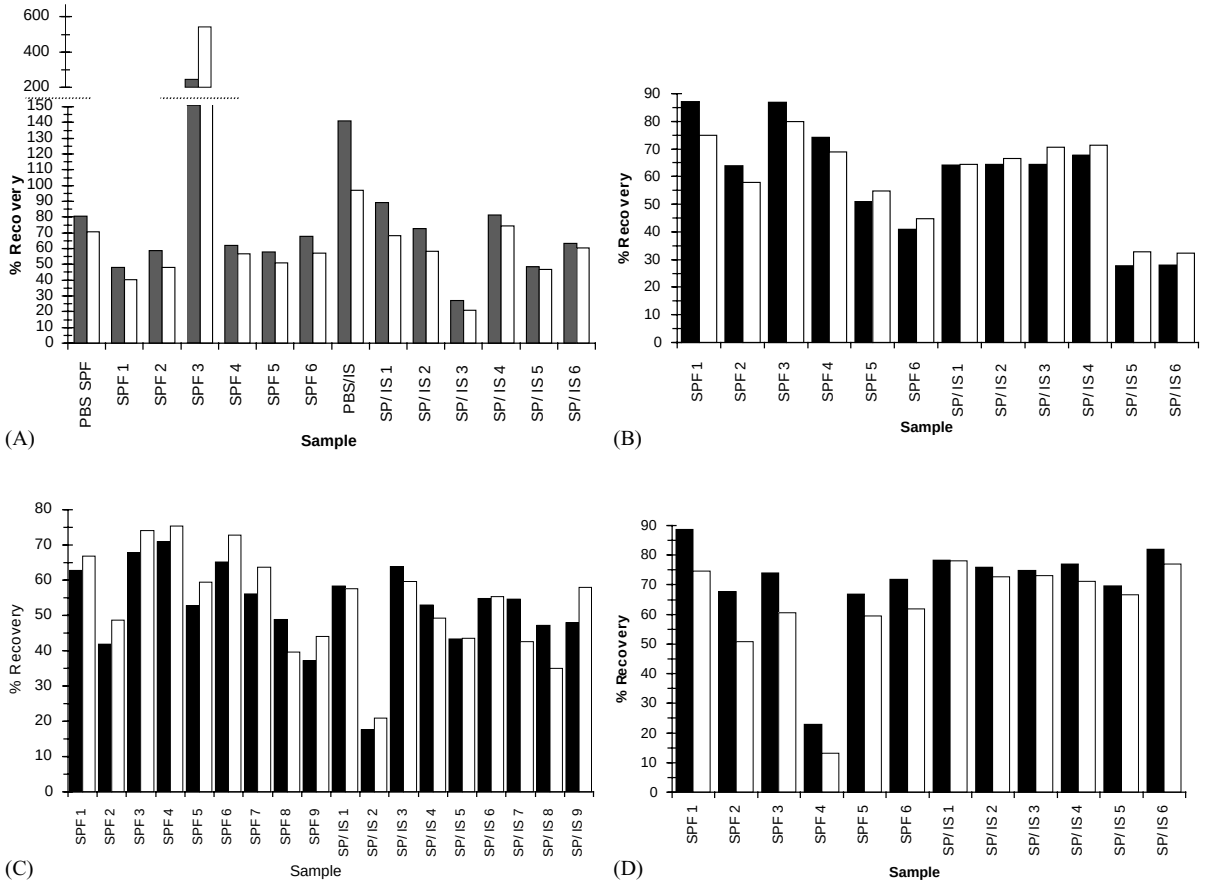

Fig. 3. Absolute extraction recovery for samples prepared in K_3EDTA human plasma (A), sodium citrate human plasma (B), sodium heparin human plasma (C) and human serum (D) (■, olanzapine-D_3; □, desmethyl olanzapine-D_8).

reduction in power for the results of lipemic category 2 and sodium heparin groups.

The percent difference in extraction recovery, matrix effects and extraction matrix effects were calculated for both internal standards. A large percent difference in matrix effect is indicative of an instrumental effect in matrix versus the control (PBS). Extraction matrix effects account for the total effect of extraction and instrumental effects in samples prepared in matrix compared to those prepared in PBS.

The percent difference in extraction recovery was calculated by comparing the difference in the response of the internal standards added prior to and post extraction. No significant effect from either lipemic category or anticoagulant was found for OLZ-D_3 ($P = 0.125$, $F = 2.16/P = 0.067$, $F = 2.79$, d.f. $= 3$) or DES-D_8 ($P = 0.046$, $F = 3.19/P = 0.051$, $F = 3.08$, d.f. $= 3$).

Variability in matrix effects was investigated by determining the differences in the response of the internal standards added post extraction into matrix extracts versus into PBS extracts (Fig. 5A and B). A significant interaction between lipemia and anticoagulant was found for both OLZ-D_3 ($P = 0.023$, $F = 3.6$, d.f. $= 8$) and DES-D_8 ($P = 0.0327$, $F = 3.3$, d.f. $= 8$). Slices were performed to determine the effect of the interaction across the lipemic categories. The most severe lipemic category 4 exhibited significantly greater matrix effects than the other categories for OLZ-D_3 ($P < 0.001$, $F(3, 12) = 21.1$, $\alpha^* = 0.00625$) and DES-D_8 ($P < 0.001$, $F(3, 12) = 18.3$, $\alpha^* = 0.00625$). Lipemic category 4 was further broken

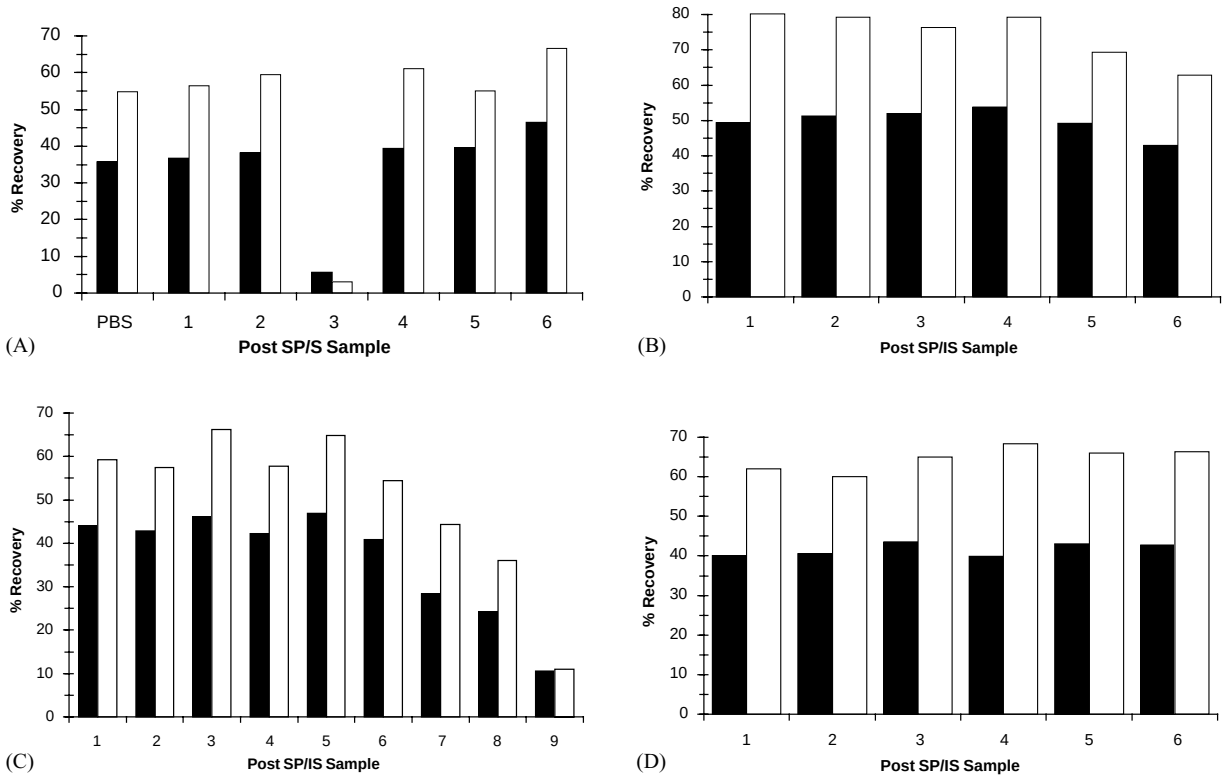

Fig. 4. Absolute instrumental recovery for samples prepared in K_3EDTA human plasma (A), sodium citrate human plasma (B), sodium heparin human plasma (C) and human serum (D) (■, olanzapine-D_3; □, desmethyl olanzapine-D_8).

down into slices to determine the effect of anticoagulant within category 4. Matrix effects on both internal standards within lipemic category 4 in plasma containing sodium heparin and K_3EDTA were significantly greater than in sodium citrate plasma or serum (all P-values ≤ 0.005, all $F(1, 12) \geq 11.8$, $\alpha^* = 0.0083$) (Table 4).

Slices were analyzed to examine the effect of the interaction between lipemia and anticoagulant across different anticoagulants. Significant matrix effects occurred with both OLZ-D_3 and DES-D_8 in plasma containing K_3EDTA (both P-values <0.001, $F(3, 12) = 21.1$ and 13.1, respectively, $\alpha^* = 0.00625$) and sodium heparin (both P-values ≤ 0.001, $F(2, 12) =$

Table 4
Statistical evidence of a matrix effect interaction between lipemic category 4 slices and anticoagulant ($\alpha^* = 0.0083$)

Comparison of	With	Olanzapine-D_3		Desmethyl olanzapine-D_8	
		$F(1, 12)$	P-value	$F(1, 12)$	P-value
K_3EDTA	Sodium heparin	7.8	0.016	8.28	0.014
	Sodium citrate	50.0	<0.001	38.6	<0.001
	Serum	25.8	<0.001	31.0	<0.001
Sodium citrate	Sodium heparin	33.2	<0.001	22.1	<0.001
	Serum	1.1	0.308	0.048	0.830
Sodium heparin	Serum	11.8	0.005	15.6	0.002

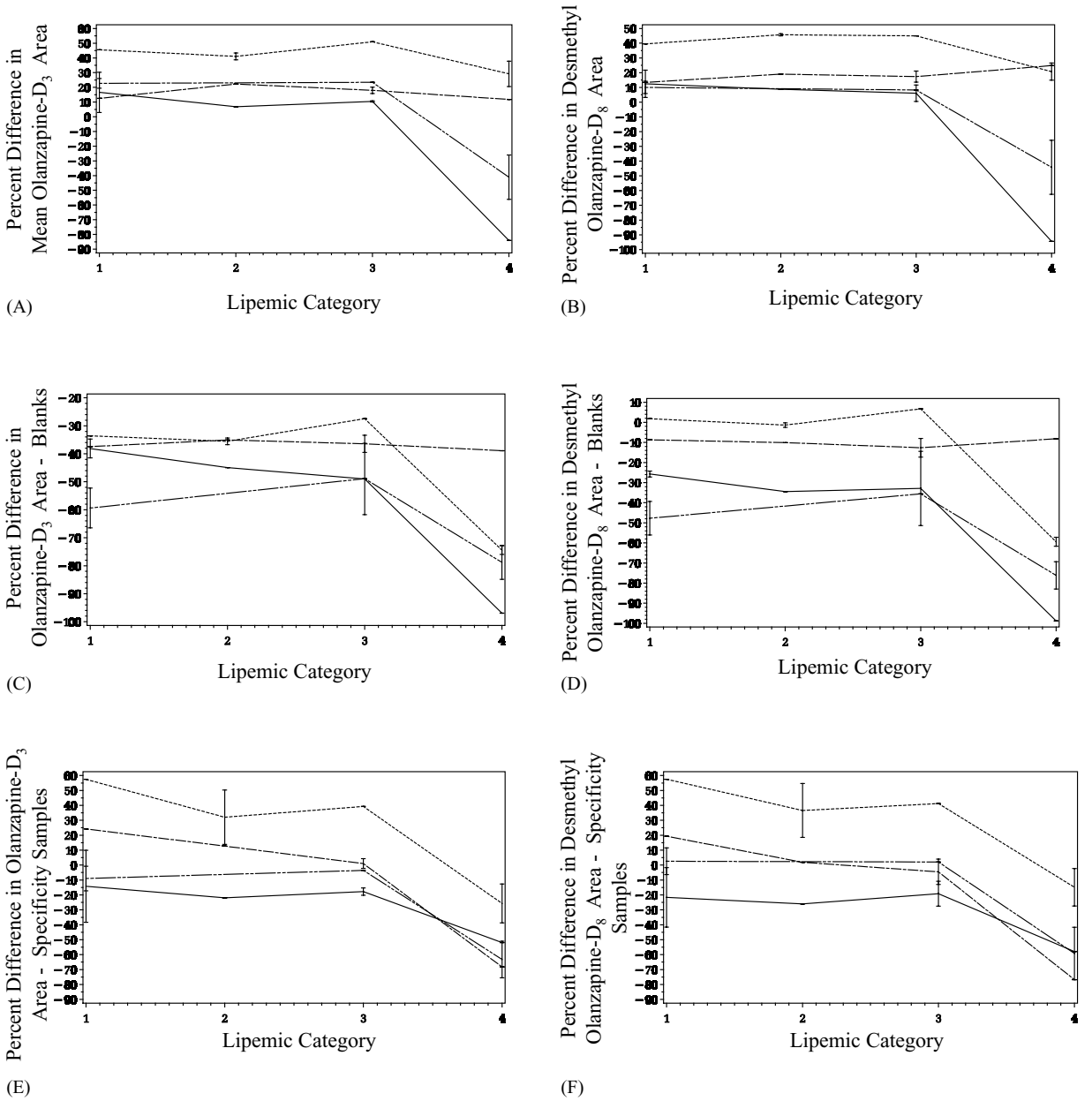

Fig. 5. Statistical analysis plots—matrix effects on olanzapine-D_3 (A), matrix effects on desmethyl olanzapine-D_8 (B), extraction matrix effects in the blanks on olanzapine-D_3 (C), extraction matrix effects in the blanks on desmethyl olanzapine-D_8 (D), extraction matrix effects in the specificity samples on olanzapine-D_3 (E), extraction matrix effects in the specificity samples on desmethyl olanzapine-D_8 (F) (—, K_3EDTA human plasma; - - -, sodium citrate human plasma; — · · —, sodium heparin human plasma; — · —, human serum).

22.9 and 12.7, respectively, $\alpha^* = 0.00625$). K_3EDTA and sodium heparin groups were further sliced to investigate the effect of lipemia within these groups (Table 5). The most severely lipemic category demonstrated significant matrix effects compared to the other categories for OLZ-D_3 and DES-D_8 in plasma containing K_3EDTA (all P-values <0.001, all $F(1, 12) \geq 23.2$, $\alpha^* = 0.0083$) and sodium heparin (all P-values

Table 5
Statistical evidence of a matrix effect interaction between K_3EDTA and sodium heparin slices and lipemic category 4 ($\alpha^* = 0.016$)

Comparison of lipemic category	With	Anticoagulant							
		K_3EDTA				Sodium heparin			
		OLZ-D_3		DES-D_8		OLZ-D_3		DES-D_8	
		$F(1, 12)$	P-value	$F(1, 12)$	P-value	$F(1, 12)$	P-value	$F(1, 12)$	P-value
4	1	37.9	<0.001	33.3	<0.001	42.8	<0.001	24.0	<0.001
	2	23.2	<0.001	23.3	<0.001	N/A	N/A	N/A	N/A
	3	33.4	<0.001	29.4	<0.001	17.5	0.001	9.00	0.011

≤ 0.011, all $F(1, 12) \geq 9.0$, $\alpha^* = 0.016$). Therefore serum or sodium citrate may be a better choice of matrix to use for an OLZ study if this assay is to be used to analyze lipemic samples.

Overall extraction matrix effects were examined by comparing the internal standard responses between matrix blanks and PBS fortified prior to extraction (Fig. 5C and D). The responses of the internal standards were compared with specificity samples prepared in matrix and those prepared in PBS (Fig. 5E and F). No significant interaction between lipemic category and anticoagulant was detected in either the blanks or the specificity samples fortified with OLZ-D_3 ($P = 0.2505$, $F = 1.5$, and $P = 0.8204$, $F = 0.5$, respectively, d.f. $= 8$) or DES-D_8 ($P = 0.1439$, $F = 1.9$, and $P = 0.9437$, $F = 0.3$, respectively, d.f. $= 8$). The model was reduced to investigate lipemia and anticoagulant separately. The most severely lipemic category 4 demonstrated significant extraction matrix effects by Tukey–Kramer HSD in both the blanks and samples for OLZ-D_3 (both P-values < 0.001, $F = 8.4$ and 18.9, respectively, d.f. $= 3$) and DES-D_8 (both P-values < 0.001, $F = 9.0$ and 17.0, respectively, d.f. $= 3$).

The overall extraction matrix effect of anticoagulant on internal standard response differed between the blanks and specificity samples. For sodium citrate, the extraction matrix effect in blanks spiked with IS prior to extraction was a negative effect but in the specificity samples, it was a positive effect. It is possible that binding to some matrix component was occurring which resulted in low extraction recovery, the addition of the analytes prior to the IS may have saturated the reaction and prevented binding of the IS. The reason for the observation of a positive effect with sodium citrate plasma may be due to the relatively lower concentration of anticoagulant in those plasmas. Sodium heparin and K_3EDTA concentrations were both lower than 5 mM however the concentration of sodium citrate was on the order of 0.2 M. Perhaps the 40-fold increase of anticoagulant concentration in sodium citrate plasma blanks prevented binding to matrix components to a greater extent relative to the other anticoagulants. This may be an explanation why sodium citrate plasma appeared to be affected less than the other groups in terms of overall extraction matrix recovery. For blanks fortified with OLZ-D_3, serum had a smaller extraction matrix effect than sodium heparin plasma ($P = 0.029$, $F = 3.7$, d.f. $= 3$). Sodium citrate plasma and serum have smaller extraction matrix effects on DES-D_8 in the blanks than plasma containing sodium heparin or K_3EDTA ($P = 0.001$, $F = 7.8$, d.f. $= 3$). In the specificity samples, the response of OLZ-D_3 ($P < 0.001$, $F = 10.2$, d.f. $= 3$) and DES-D_8 ($P < 0.001$, $F = 11.4$, d.f. $= 3$) were affected differently in sodium citrate plasma than in all of the other anticoagulated plasmas and serum. Sodium citrate specificity samples were influenced positively while K_3EDTA, sodium heparin and serum were affected negatively. The absolute mean of the sodium citrate group was within the range of the absolute means of the other groups, therefore it is difficult to judge the magnitude of the effect on the specificity samples which were prepared in sodium citrate plasma. The statistical results are summarized in Tables 6–8.

3.3. Ion suppression experiments

Based on low recovery and high variability, problem samples were identified for further testing including ion suppression experiments. Ion suppression profiles were determined for a number of samples through

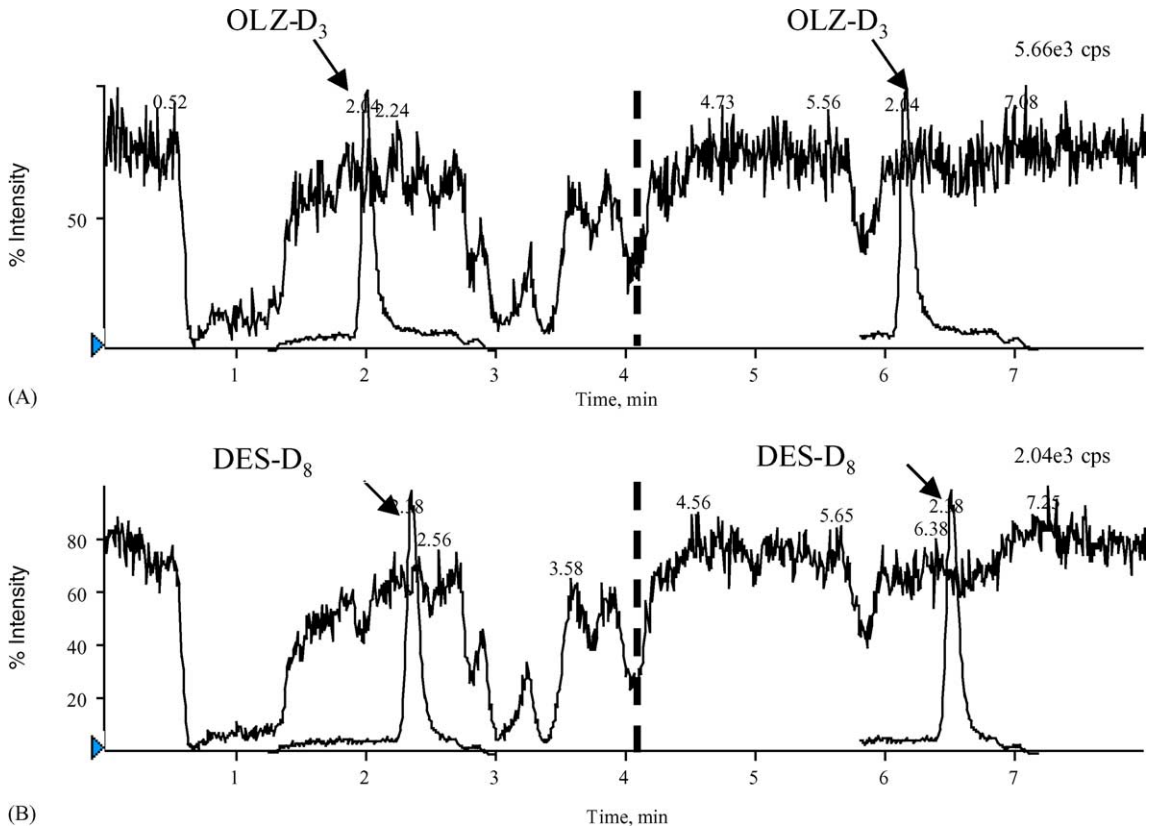

Fig. 6. Ion suppression profiles of an extracted sodium heparin human plasma blank fortified with internal standard and injected under isocratic conditions (98% mobile phase A acetonitrile–ammonium acetate (20 mM) (52:48 v/v) and 2% mobile phase B formic acid–acetonitrile (0.1:100 v/v); flow rate = 0.4 ml/min). The dotted vertical lines represent the starting point of the subsequent injection. Internal standard chromatograms were overlaid to indicate approximate retention times. (A) Olanzapine channel, (B) desmethyl olanzapine channel.

post column infusion experiments. The elution of polar compounds at the solvent front and changes in the mobile phase by the gradient were expected to cause depressions in the baseline signal but other areas of suppression were also observed. The original method was validated under isocratic chromatographic conditions. During preliminary ion suppression experiments, it was determined that under isocratic conditions late ion suppression peaks elute in close proximity to the retention times of interest in the subsequent injection (Fig. 6). A slight shift in the retention times of OLZ, olanzpine-D_3 or the ion suppression peaks could result in co-elution. Therefore gradient conditions were investigated in an attempt to resolve the late eluting suppression from the retention times of interest in the next injection (Fig. 7). The late eluting suppression was shifted earlier and became less of a hazard through the modification of the chromatographic conditions.

Even after the chromatographic conditions were modified to include a gradient, the responses of the analytes and internal standards were significantly re-

Table 6
Summary of extraction matrix effects in relation to lipemic category

Lipemic category	Olanzapine-D_3	Desmethyl olanzapine-D_8
1	No effect	
2	No effect	
3	No effect	
4	Overall extraction matrix effect	

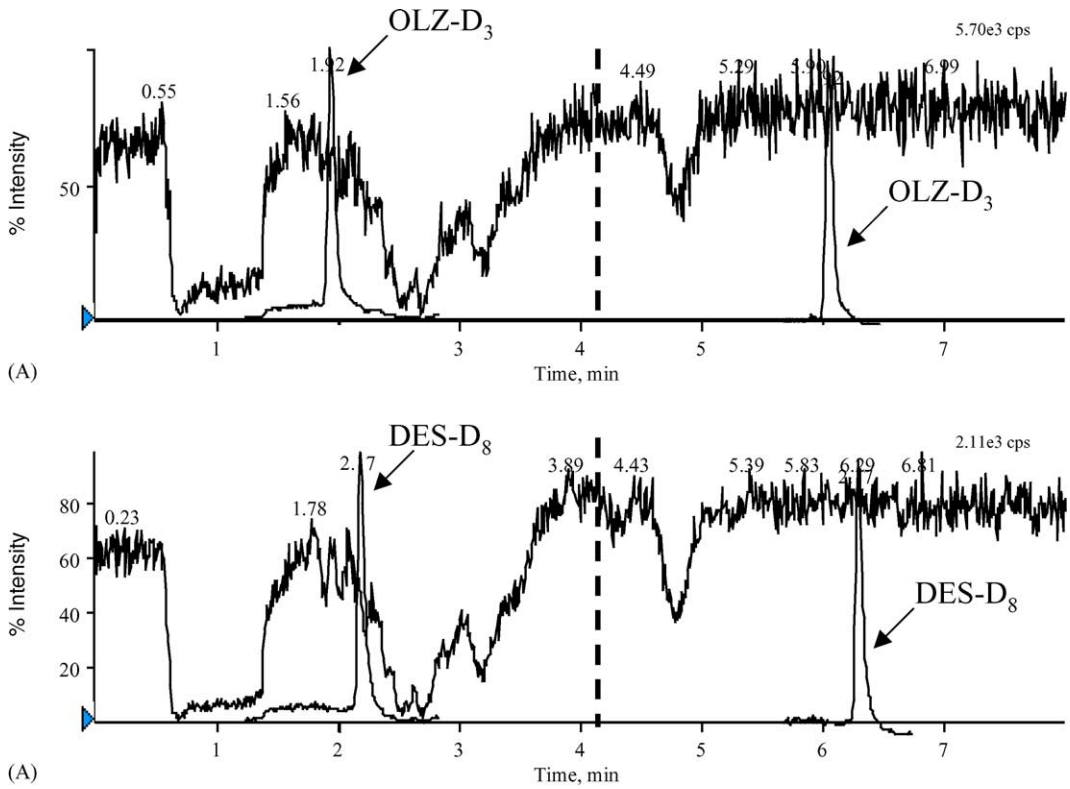

Fig. 7. Ion suppression profile of an extracted sodium heparin human plasma blank fortified with internal standard and injected under gradient conditions. The dotted vertical lines represent the starting point of the subsequent injection. Internal standard chromatograms were overlaid to indicate approximate retention times. (A) Olanzapine channel, (B) desmethyl olanzapine channel.

Table 7
Summary of extraction matrix effect on blanks in relation to anticoagulant

Anticoagulant	Olanzapine-D_3	Desmethyl olanzapine-D_8
K_3EDTA	No effect	Sodium citrate and serum < sodium heparin and K_3EDTA
Sodium citrate	No effect	
Serum	Serum < sodium heparin	
Sodium heparin	Serum < sodium heparin	

Table 8
Summary of extraction matrix effect on specificity samples in relation to anticoagulant

Anticoagulant	Olanzapine-D_3	Desmethyl olanzapine-D_8
K_3EDTA	Affected negatively	
Sodium citrate	Affected positively	
Serum	Affected negatively	
Sodium heparin	Affected negatively	

duced by ion suppression. The isotopically labeled internal standards were not able to correct for the effect of ion suppression. When samples fortified with the internal standards post extraction were analyzed, up to 90% reduction in the response of both internal standards was observed. An indication of possible problems with ion suppression is a reduction in the response of the internal standards from a matrix extract compared to a water extract. Matrix components present in the plasma or serum extracts

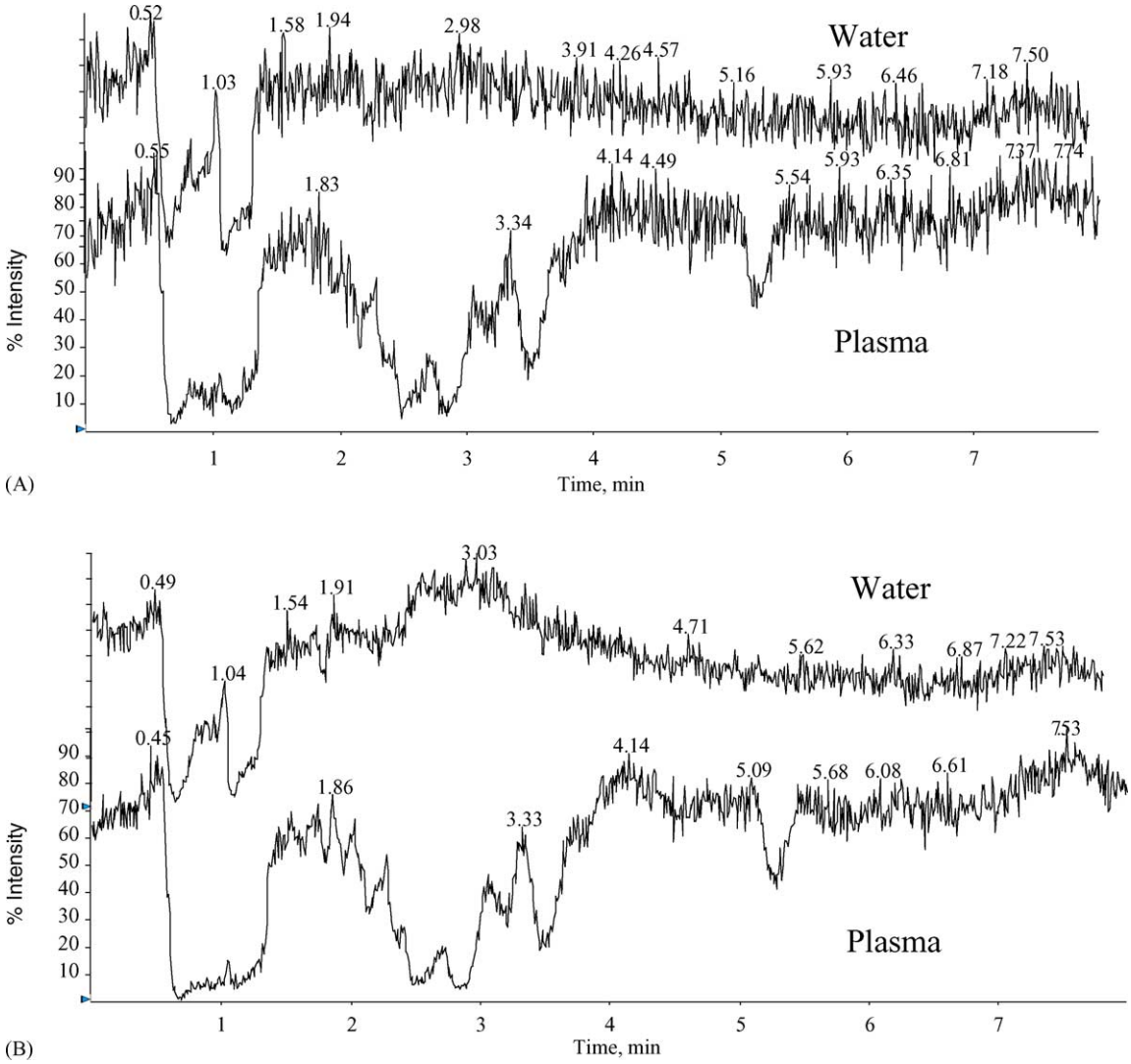

Fig. 8. Ion suppression profiles of water and sodium heparin human plasma blank extracts fortified with internal standard. (A) Olanzapine channel, (B) desmethyl olanzapine channel.

can cause additional ion suppression compared to a water extract (Fig. 8). Polar components eluted early, t_R = 0.5–1.5 min, with the solvent front causing the first decrease in signal. There were no observable differences in the profiles of water and PBS which suggests that salt content was not a major factor (data not shown). The second broad band of suppression present in the plasma extract (Fig. 9), t_R = 1.5–4.0 min, was due to the elution of matrix components. Since the analytes and internal standards co-eluted with this suppression their responses were significantly reduced (Fig. 7). This broad band of suppression accounted for approximately 10–25% of the reduction of response.

The runtime of each injection was doubled to 8 min during the ion suppression experiments in order to detect any late eluting suppression that may affect a subsequent injection. A third smaller band of suppression, t_R = 5.1–5.5 min, was found and could have potentially affected the response of the analytes and

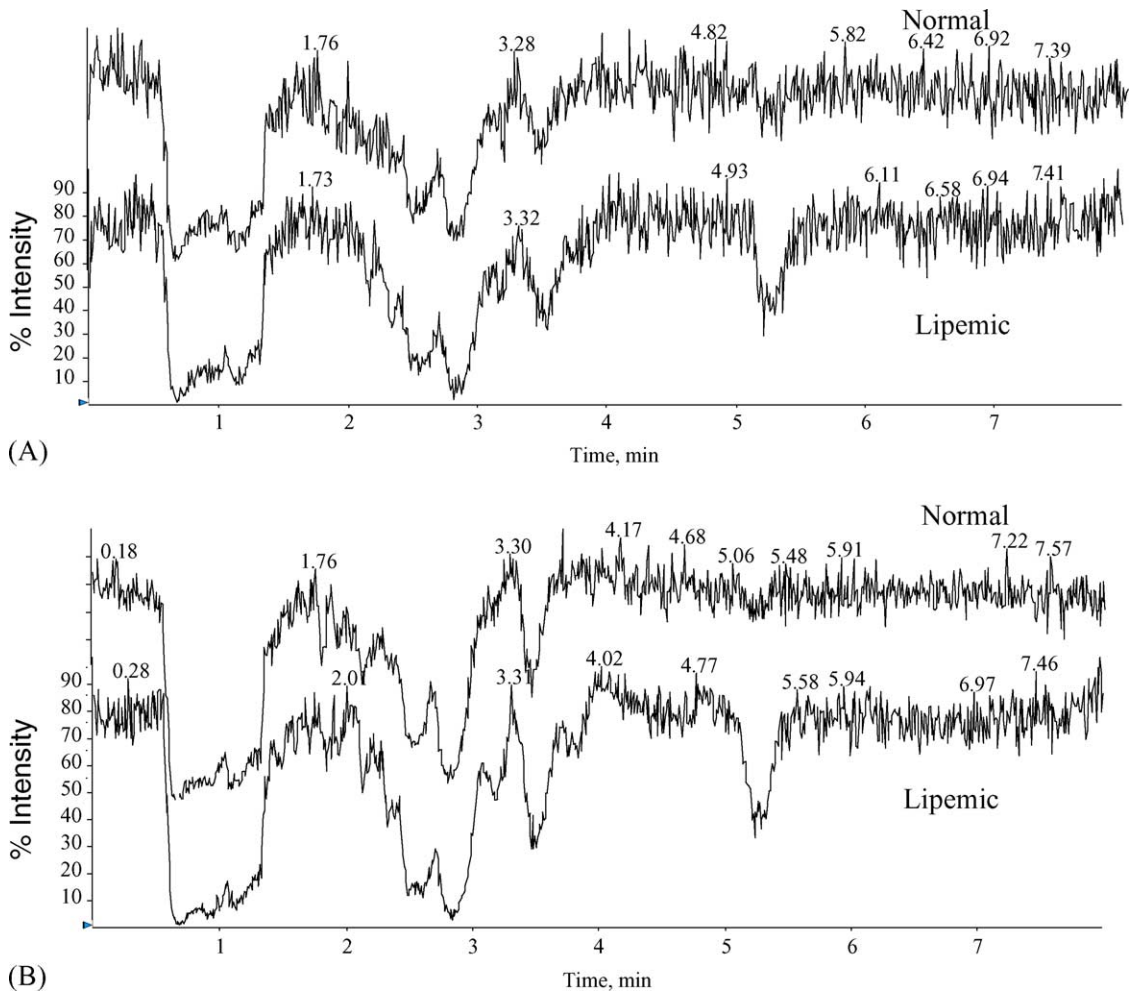

Fig. 9. Ion suppression profiles of normal and lipemic K_3EDTA plasma. (A) Olanzapine channel, (B) desmethyl olanzapine channel.

internal standards in the following injection assuming a runtime of 4:10 min. The late band of suppression was more apparent in lipemic samples compared to normal samples (Fig. 8). In lipemic samples, the late band of suppression accounted for the reduction of approximately 40–50% of the response of the analytes and internal standards. Even if the worst case scenario was assumed and both the broad band and the late suppression simultaneously affected the overall response of the analytes and internal standards, ion suppression could not account for the over 90% reduction in signal observed in several samples. Therefore ion suppression was determined not to be solely responsible for the variability of the specificity results but instead recognized as a major contributor. Differences in column performance between different lots of LUNA phenyl hexyl columns also affected assay performance due to changes in retention time, which was a major determinant in the degree of ion suppression observed.

Extraction consistency also contributed to the variability of the ion suppression profiles. The ion suppression profiles from replicates aliquoted from the same sample tube and extracted on the same plate were associated with a great deal of variability (Fig. 9). The degree of ion suppression in replicate 1 was substantially less than in replicate 2. Therefore variability in the extraction of suppressing matrix components may

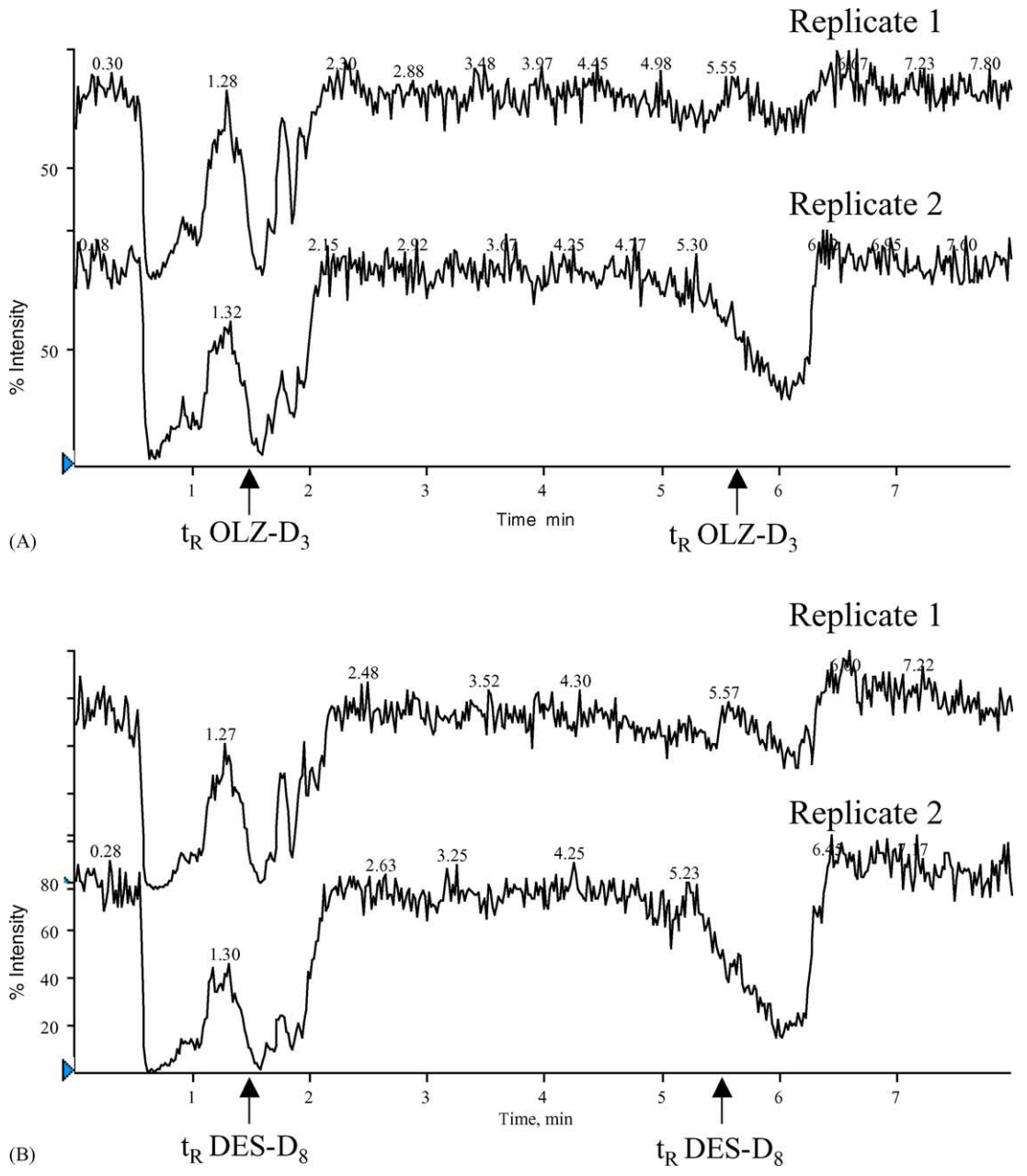

Fig. 10. Ion suppression profiles of multiple replicates of K_3EDTA plasma blank extracts. (A) Olanzapine channel, (B) desmethyl olanzapine channel.

cause inconsistent ion suppression profiles. The poor reproducibility of the ion suppression profiles between replicate extracts made it difficult to accurately correlate the degree suppression with either anticoagulant or lipemia. Many unsuccessful attempts were made to alter the chromatographic conditions, through modification of the mobile phase and gradient programs. Alternative extraction procedures, such as liquid–liquid extractions, and SPE using C_{18} and C_8 cartridges were also attempted with unsatisfactory results (Fig. 10).

4. Conclusions

Obtaining reliable results from this assay for OLZ and DES was compromised by several problems. Differential extraction behavior between the analytes and isotopically labeled internal standards caused variability issues with quantitation. The differential behavior may be due to a reaction, which occurs during storage of the samples, between the analytes and matrix components present, particularly in lipemic samples, which sequester the analyte and prevent its release during extraction. Ion suppression, caused by co-eluting matrix components, was a major determinant of overall and instrumental recovery and accounted for ∼20–95% of the reduction in the response of the analytes of interest. The ion suppression was unable to be chromatographically resolved from the retention times of the analytes and internal standards. Differences in the extraction of matrix components resulted in variable suppression profiles which contributed to intra-sample variability. Lastly, anticoagulant and lipemia were determined to significantly affect instrumental matrix and overall extraction matrix effects.

The results indicate that sodium heparin and K_3EDTA human plasma are probably not the best matrix to use for a clinical OLZ study. This assay should not be used to quantitate OLZ and DES in lipemic samples. The extraction procedure and chromatographic conditions should be modified to correct these problems if lipemic samples are to be analyzed. The possibility of complex formation between the analytes and matrix components should also be further investigated. In general, these results suggest that matrix effects, due to matrix components such as anticoagulant and lipids, affect the performance of an assay significantly and should be evaluated during a validation or prior to the analysis of patient samples.

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