Journal of Forensic Research
ISSN: 2157-7145

Zubaidi et al., J Forensic Res 2019, 10:1
DOI: 10.4172/2157-7145.1000436

Research Article Open Access

Bland-Altman Statistical Analysis and Mean Concentration Ratio for the Determination of Amphetamine-type Stimulants-related Drugs in Dried Blood Stain (DBS) Versus Whole Blood Sample (WBS) by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Application to Forensic Toxicology Cases in Malaysia

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Received date: March 11, 2019; **Accepted date:** April 3, 2019; **Published date:** April 10, 2019

Abstract

The performance of dried blood stain (DBS) versus whole blood sample (WBS) methods were evaluated using Bland-Altman analysis and mean concentration ratio for forensic toxicology cases in Malaysia. Common amphetamine-type stimulants related drugs of ephedrine, pseudoephedrine, amphetamine, methamphetamine, MDMA (3,4- Methylenedioxymethamphetamine), MDA (3,4-Methylenedioxyamphetamine), MDEA (3,4-Methylenedioxy-N-ethylamphetamine) and phentermine were investigated using DBS and WBS methods. The analysis was performed utilizing novel protocol consists of mass spectrometry detection technique based on a multi-period and multi-experiment (MRM-EPI- MRM3) with library matching post liquid-liquid extraction of whole blood and corresponding DBS samples from spiked samples. The analysis was applied to 20 whole blood samples submitted for forensic and medico-legal analysis which were reported positive for the presence of the drugs. Results for both DBS and WBS were compared using Bland-Altman mean difference plots and mean concentration ratio for degree of agreement. The results showed good degree of agreements indicating no significant differences in results obtaining from DBS and WBS methods, allowing both methods to be used interchangeably. The study also demonstrated the advantage of the DBS method as an inexpensive alternative to WBS in the forensic toxicology field.

Keywords: DBS; WBS; Bland-Altman; Degree of agreement; LC-MS/MS

Introduction

The use of dried blood stain (DBS) as an alternative matrix has been gaining popularity as samples can be collected easily with minimum chances for adulteration, greater stability over other matrices including blood, as well as have the potential to identify recent drugs consumption [1-5]. Although DBS has been gaining acceptance in therapeutic drug monitoring in recent years, however, applications to forensic samples have not received similar attentions.

In the clinical and forensic studies, a newly developed method needs to be compared against an established method to determine whether these methods can be used interchangeably or the new method can replace the established method [6-9]. In most cases, the 'true' value of the measured quantity is unknown.

Investigations of DBS and WBS are often analysed by using correlation coefficients, r which measures the strength of a relation between two variables, not the agreement between them [10-12]. The magnitude of the correlation coefficient can be reduced almost equal to 1 by measuring samples that are similar to each other and larger by measuring samples that are very different from each other. Hence, the magnitude of the correlation does not indicate the differences between the two methods being measured.

Bland-Altman statistical analysis compares two methods of measurement to determine 1) interchangeable between the methods and 2) replacement of old method by new method [6,7]. The advantages of using Bland-Altman plot compare to other type of statistical analysis such as t-test and correlation coefficient is that Bland-Altman uses graphical techniques and simple calculations to measure the mean difference, relation between magnitude of the compared analysis and the assessment of repeatability, in which t-test and correlation coefficient method can only measure one parameters at one time.

The new method has to be evaluated by comparing the technique with true quantity. In most cases, the 'true' value of the measured quantity is unknown. If the new method agrees sufficiently with the existing method, hence, it is possible to replace the existing method with the new method. In cases where the comparison of two methods does not provide obvious comparable measurements, the degree of agreement will be assessed [9,10].

The limit of agreement allows estimation of the closeness between the new and old method measurements, carried out by the same analyst. If these limits are within satisfactorily limits and suggested similar conclusions about the measured quantity, it can be concluded

that the methods agree sufficiently well for the two methods to be used interchangeably [11].

The Bland-Altman method calculates the mean difference between two methods of measurement with 99% limits of agreement as the common mean difference (3SD) or more precisely (2.575 SD). It is estimated that the 99% limits include 99% of differences between the two measurement methods. The Bland-Altman plots show the difference between method values (y-axis) against the average of method values (x-axis), and provide an assessment of the level of agreement between the two methods [12]. The presentation of the 95% limits of agreement is for visual judgement on how well the measurement of the two methods agrees. The smaller the range between these two limits the better is the agreement [13].

Several successful applications of the Bland-Altman comparison methods included the detection of low level of lead in child whole blood in clinical setting using LeadCare® System (LCS) to exchange the 'gold standard' of Inductively Coupled Plasma Mass Spectrometry (ICP-MS) [14]. LCS was reported to be comparable to the ICP-MS method, and hence, demonstrated the device suitability for the clinical evaluation and monitoring of blood lead levels among individual children.

Mean concentration ratio, t-test and Bland-Altman [1] methods have been applied in the evaluation of DBS versus WBS for the determination of 3,4- methylenedioxymethamphetamine (MDMA) and its main metabolite 3,4- methylendioxyamphetamine (MDA) in a controlled driving experiment under the influence of MDMA. Statistical analyses revealed that the bias in mean concentration values were too small and methods did not show significant differences for MDMA nor MDA, proving the reliability and potential of the precise and inexpensive DBS method as an alternative to WBS analysis of MDMA.

The aim of this study was to compare the performance of dried blood stain (DBS) versus whole blood sample (WBS) methods using Bland-Altman analysis and mean concentration ratio for the determination of ATS-related drugs from multiple reaction monitoring (MRM), enhanced product ion (EPI) and multiple reaction monitoring with multistage fragmentation (MRM3) (MRM-EPI-MRM3) spectrometry analysis. The investigation was extended to real forensic toxicology cases in Malaysia. The comparison was to determine the quantitative analysis of the drugs using DBS method is equivalent and has potential to be an alternative to WBS method.

Materials and Methods

Materials

All certified reference materials (CRMs): amphetamine (AMP), methamphetamine (MA), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxy-N-methylamphetamine (MDMA), 3,4-methylenedioxy-N-ethylamphetamine (MDEA), pseudoephedrine (PEP), ephedrine (EP), phentermine (PTM), methamphetamine-d14 (MA-d14) and 3,4-methylenedioxy-N-methylamphetamine-d5 (MDMA-d5) were purchased from Lipomed (Switzerland). Acetonitrile and methanol were HPLC grade (Merck, Darmstadt, Germany). Chlorobutane was acquired from Fischer Chemical (Loughborough, Leicestershire, UK). Formic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). Whatman® FTA® card was purchased from Merck (Darmstadt, Germany). Ultrapure water was from arium® pro UV Ultrapure Water with a specific resistance at >18 MΩcm. Phosphate buffer (20 mM, pH 7.4) was prepared by dissolving monobasic sodium phosphate in water, followed by adding sodium hydroxide to adjust the pH.

Drug-free whole blood was obtained from bull's blood that has been tested earlier and was used in the preparation of calibration for matrix matched analysis. Whatman FTA Card was selected as DBS medium. External Quality Controls (QC) consisted of whole blood sample tested for Proficiency Testing provided by College of American Pathologists (CAP) that had been accredited under ISO 17025 and International American Society of Crime Lab Directors-Laboratories Accreditation Board (ASCLD-LAB) accreditation.

Authentic samples

Whole blood and corresponding DBS samples (n=20) with positive identification of ATS-related drugs were obtained from various real cases submitted for forensic and medico- legal analysis from January 2016 until September 2017 to Government Enforcement Laboratory of Forensic Division, Department of Chemistry, Malaysia. Blood stains were prepared by spotting 100 µL aliquot of blood onto Whatman FTA Card, which were subsequently dried at room temperature overnight. All samples were stored at 4°C until the time of analysis. Analysis was performed by LC-MS/MS following liquid-liquid extraction of both medium.

Analysis of ATS-related drugs from DBS and WBS by LC-MS/MS

Calibration solutions for AMP, MA, MDA, MDMA, MDEA, PEP, EP and PTM were prepared in WBS and DBS at six different concentrations of 5, 10, 20, 50, 100 and 200 ng/mL. Seven replicates with concentration of 20 and 100 ng/mL were also prepared in both medias for method validation.

WBS was subjected to liquid-liquid extraction (LLE). An aliquot (30 µL) of the internal standard mixture (MA-d14 and MDMA-d5) solution was added into 1 mL of the samples, followed by 0.5 mL of phosphate buffer solution and 3 mL of 1-chlorobutane. The samples were then equilibrated on a roller mixer for approximately 1 hour followed by centrifugation at 2000 rpm for 10 mins. The upper organic solvent layer was transferred to a clean tube and dried using rotary evaporator. The dried extract was re-constituted in 80 µL of 50% methanol solution and transferred to an autosampler vial.

DBS were cut out with a puncher and transferred into test tubes followed by adding 30 µL of the internal standard mixture (MA-d14 and MDMA-d5) solution. LLE was performed by adding 1 mL of water followed by 0.5 mL phosphate buffer (phosphate buffer releases Hb from the stain and lead to best recovery) and 3 mL of 1-chlorobutane. The subsequent steps followed that of WBS accordingly for LC-MS/MS analysis.

LC-MS/MS analysis was performed on an Exion LC SCIEX Binary SL Series System (Toronto, Canada) consisting of an autosampler, a binary pump and a column for chromatography. LC separation was performed by injecting 5 µL of samples on a reversed- phase C18 Luna Omega analytical column (100 mm X 2.1 mm X 1.6 µm particle size; Phenomenex, USA). The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B).

For the MS conditions, a SCIEX 5500 hybrid QTRAP tandem mass spectrometry system (Toronto, Canada) equipped with patented Turbo V source was used. Compound ionisation was performed using

electrospray ionisation (ESI) and set at positive mode. Analyst software version 1.6.3 together with MultiQuant version 3.0 was used during method development, data acquisition, data processing and statistical analysis. Further assessment for Bland-Altman plot [15] was done by Microsoft Excel.

Data were monitored with the following transitions: AMP m/z 136.1 → 119.1* and 136.1 → 91.1; MA m/z 150.1 → 119.1* and 150.1 → 91.1; MDA m/z 180 → 105* and 180→ 133; MDMA m/z 194 → 163.1* and 194 → 105.1; MDEA m/z 208.1 → 163* and 208.1→ 135; PEP/EP m/z 166.2 → 148.2* and 166.2 → 115.2 (pseudoephedrine, PEP and ephedrine, EP were determined *via* chromatographic separation); PTM m/z 150.1 → 133* and 150.1 → 91.1; MA-d14 m/z 164 → 98 and MDMA-d5 m/z 199 → 165 (Transitions marked with an asterisk were used for quantitation and Bland-Altman analysis).

Calibration curves were constructed using the peak area ratio of the analyte and internal standard, plotted versus the corresponding concentration and determined by linear regression with a 1/x weighting factor. The reagent-only calibration standards and matrix-matched calibration standards were used to assess the matrix effects as described by Matuszewski et al. [16].

Parameters including linearity, limit of detection (LOD) and limit of quantification (LOQ), accuracy and precision (within the laboratory repeatability and/or within the laboratory reproducibility conditions) were also investigated to determine robustness and carry over following guidelines from the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology [17] and UNODC Guidance for the Validation of Analytical Methodology and Calibration of Equipment used for Testing of Illicit Drugs in Seized Materials and Biological Samples [18].

Results

Evaluation of the analytical assay

Method validation showed no detection of interfering peaks from endogenous compounds at the retention times of the analytes from the blank matrix and no carryover. The results from matrix effect evaluation was 80%-110% with coefficient of variation (CV) less than 10% for the entire set of analytes. The good performance was partially attributed to the addition of the isotopically labelled internal standards coupled with the highly selective MRM3 mode. Linearity was assessed based on the six concentration levels of each analytes and the results showed good linear relationships with correlation coefficients greater than 0.994 for all targeted analytes in both medium.

The overall recoveries were within 15% range of the target concentrations with standard deviations below 9%. The intra-day and inter-day precision results have shown acceptable precision ranging from 4.62 to 7.80% (% RSD) in WBS, and 2.71 to 8.88% in DBS. The findings revealed good precisions and accuracies as well as confirmed the robustness of the method.

Drugs	EPHED - WBS	EPHED - DBS	PSEUDO- WBS	PSEUDO- DBS	AMPHE - WBS	AMPHE - DBS	MDA-WBS	MDA-DBS	METH-WBS	METH-DBS	MDMA - WBS	MDMA - DBS	PHENT - WBS	PHENT - DBS	MDEA - WBS	MDEA - DBS
Mean (pool of 21 data)	20.067	19.112	18.189	19.48	19.575	18.723	19.538	19.506	20.476	20.507	18.947	20.762	19.597	20.139	21.463	21.122
Mean Concentration ratio	1.05	1.05	0.934	0.934	1.046	1.046	1.002	1.002	0.998	0.998	0.911	0.911	0.973	0.973	1.016	1.016

Table 1: Mean concentration ratio of 20 ng/mL, pooled from 21 spiked for WBS and DBS.

Drugs	EPHED-WBS	EPHED-DBS	PSEUDO- WBS	PSEUDO- DBS	AMPHE-WBS	AMPHE- DBS	MDA-WBS	MDA-DBS	METH - WBS	METH - DBS	MDMA - WBS	MDMA - DBS	PHENT-WBS	PHENT- DBS	MDEA - WBS	MDEA - DBS
Mean (pool of 21 data)	96.426	93.607	95.44	98.631	100.2	100.063	97.786	98.483	93.236	102.185	99.846	97.362	96.085	95.737	100.089	100.705
Mean Concentration ratio	1.03	1.03	0.968	0.968	1.001	1.001	0.993	0.993	0.912	0.912	1.026	1.026	1.004	1.004	0.994	0.994

Table 2: Mean concentration ratio of 100 ng/mL, pooled from 21 spiked for WBS and DBS.

Mean concentration ratio for WBS versus DBS

The mean concentration ratio of 1.0 between two methods of measurements indicates the exact agreement of both methods [1]. The mean ratio of ATS-related drugs concentrations for WBS/DBS was determined at 20 and 100 ng/mL. The study revealed WBS/DBS ratios ranged from 0.91 to 1.05 for all targeted analytes suggesting that the two methods were comparable and the low relative standard deviation (% RSD) of 0.97 to 2.33 indicates insignificant differences between the WBS and DBS values (Tables 1 and 2).

Bland-Altman analysis for WBS versus DBS

Bland-Altman emphasizes on the comparative agreement rather than correlation coefficient and linear regression for the degree of agreement between two methods as both correlation coefficient and linear regression measurements have its shortcoming. The correlation coefficient and linear regression values can be as close as 1.0 with presence of significant bias in the measured methods. For example, regression value can be closed to 1.0 in cases where calibration measurement is 7 units higher than the intended value, as long as the

minimum points are considered for calibration curve. As a consequence, the significant bias cannot be assessed accurately when using Bland-Altman analysis.

The preeminent way to use Bland-Altman plot system is to define the bias, limit of acceptable difference (limits of agreement), and determine whether the data points are within or exceed the stated limits. In order to do so, the mean difference of each drugs analyzed in WBS *vs* DBS was calculated. Bias of the average difference of mean and the standard deviation was also determined. 99% confidence interval and its standard deviation (SD) was calculated prior to determining the lower limit of agreement and the upper limit of agreement; Bias + 3SD and Bias-3SD.

The 99% confidence interval of mean difference and of the agreement limits were used in this study to described possible errors in the estimation, e.g. from sampling and analysis. The greater the number of samples used for the evaluation of the difference between the methods, the narrower the confidence interval, for the both mean difference and limits of agreement.

Following Bland-Altman method, the SD of the difference between the means of the repeated measurements was calculated based on the within-subject. The Bland-Altman plot for difference of WBS-DBS *vs* mean difference as its y-axis and x-axis, respectively was constructed. Bias, lower limit, upper limit and each data point for the corresponding drugs was also plotted. The Bland-Altman plot showed the difference between individual measurement and the average of the means. The SD difference for each drug in WBS and DBS was further calculated using Microsoft Excel 2016.

Discussion

In this study, the Bland-Altman plots were constructed for the spiked samples and real cases samples that were reported positive for the presence of ATS-related drugs from Government Enforcement Laboratory, Forensic Division of Department of Chemistry, Malaysia together with the external QCs from International Proficiency Testing of College of American Pathologists (CAP), USA. For the spiked samples, low and high level of ATS- related drugs spiked into the WBS and DBS were analyzed i.e., 20 and 100 ng/mL, respectively.

Results from Bland-Altman difference plots (Figures 1-3) suggested that ATS-related drugs could be identified and quantified from DBS as precisely as WBS for forensic interests.

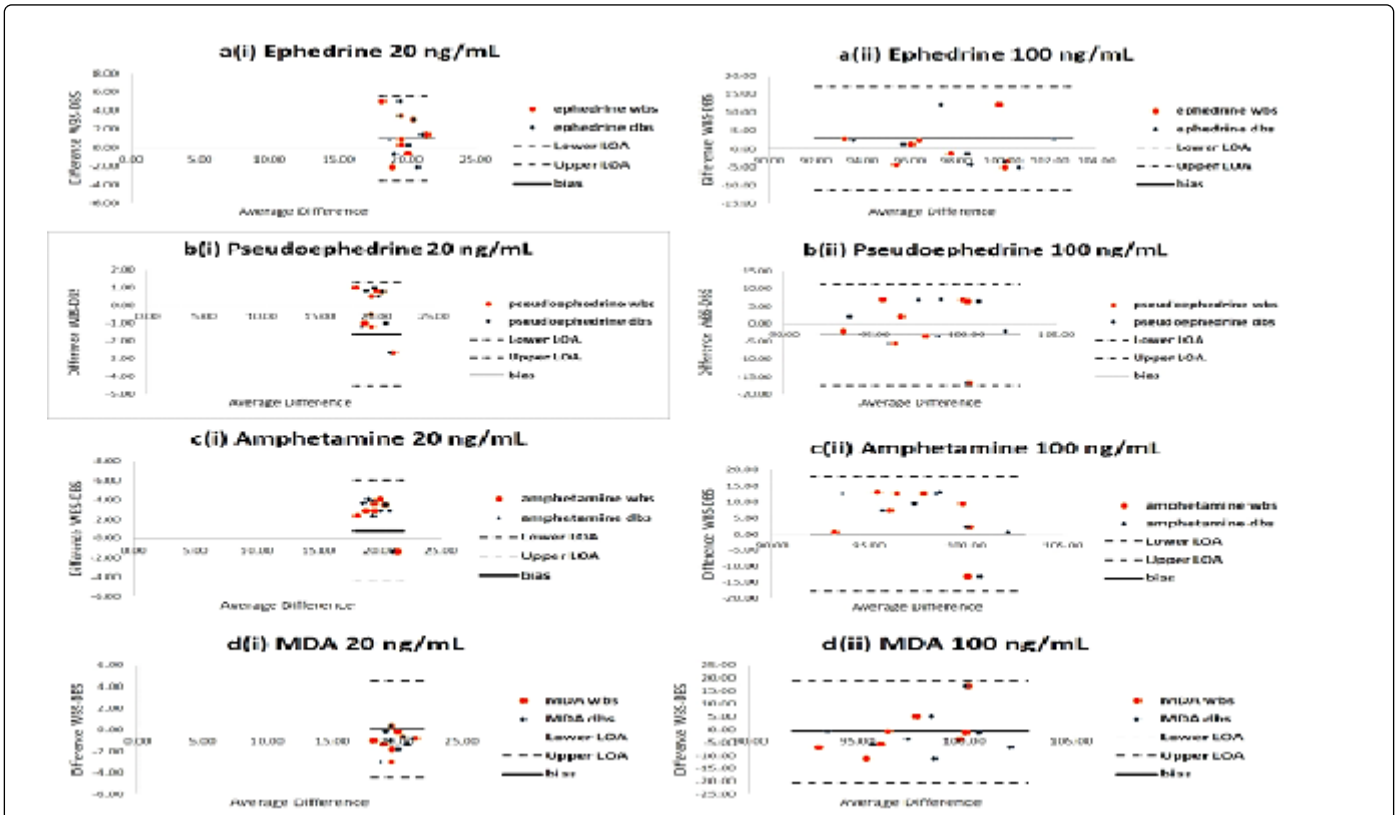

Figure 1: Bland-Altman difference plots [a(i) and a(ii)-Ephedrine; b(i) and b(ii)-Pseudoephedrine; c(i) and c(ii)-Amphetamine; d(i) and d(ii)-MDA] of the differences between whole blood specimen (WBS) and dried blood stain (DBS) method respectively, against the average obtained by the two assays for ATS-related drugs (spiked at 20 and 100 ng/mL). The solid lines illustrate the mean differences; the dotted lines indicate the limits of agreement. (Note: Lower LOA (bias-2.575 SD), Upper LOA (bias+2.575 SD)).

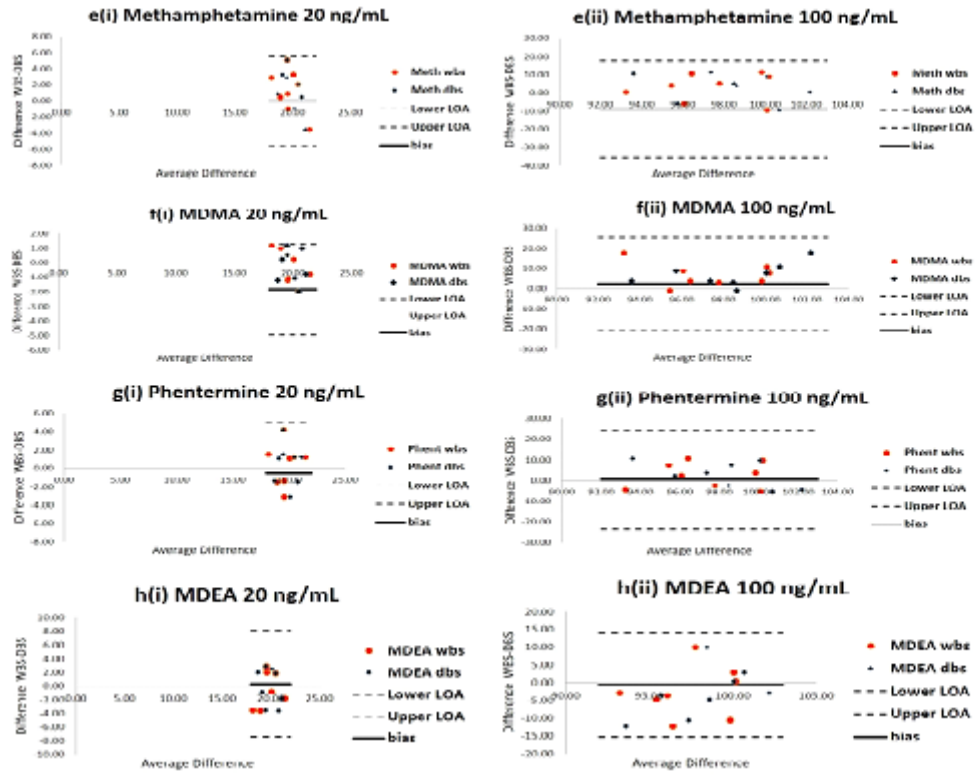

Figure 2: Bland-Altman difference plots [e(i) and e(ii)-Methamphetamine; f(i) and f(ii)-MDMA; g(i) and g(ii)-Phentermine; h(i) and h(ii)-MDEA] of the differences between whole blood specimen (WBS) and dried blood stain (DBS) method respectively, against the average obtained by the two assays for ATS-related drugs (spiked at 20 and 100 ng/mL). The solid lines illustrate the mean differences; the dotted lines indicate the limits of agreement. (Note: Lower LOA (bias-2.575 SD), Upper LOA (bias+2.575 SD)).

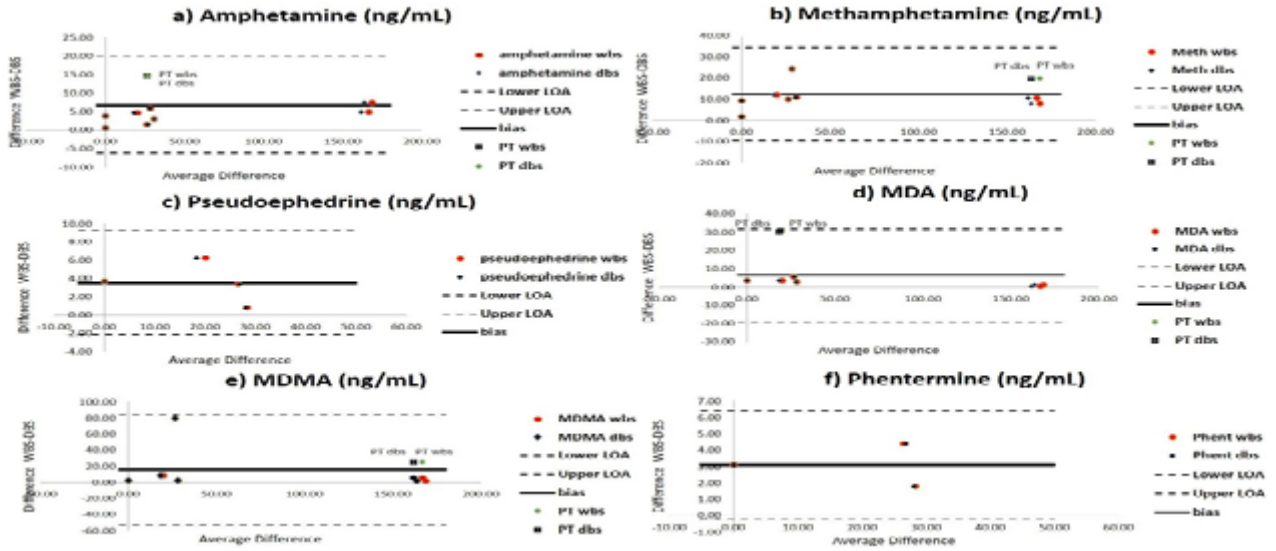

Figure 3: Bland-Altman difference plots (a) Amphetamine, (b) Methamphetamine, (c) Pseudoephedrine, (d) MDA, (e) MDMA, (f) Phentermine of the differences between whole blood specimen (WBS) and dried blood stain (DBS) method against the average obtained by the two assays for ATS-related drugs in 20 real cases samples and 4 external QCs (CAP, USA).

All data points tested were within limits of agreement and 99% of the points were within 3SD of the mean difference. The results suggested that such in the long run, 99% of future mean differences between measurements made on the same instrument will also lie within the limit of agreement.

Conclusion

In this study, we have demonstrated the use of mean ratio and Bland- Altman plots to test hypothesis of equality between two mediums; i.e. WBS *vs* DBS, in the analysis of ATS- related drugs. Estimation of the both mediums using the methods have shown that the methods were not significantly different from each other. The two methods agreed sufficiently well for them to be used interchangeably. The results also demonstrated that DBS would be a potential alternative to WBS in forensic toxicology field.

Acknowledgment

This work is partially supported by the Toxicology Division, Forensic Science Analysis Centre, Department of Chemistry, Malaysia (MESTECC).

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