Original Research Article

Validation of the Alere™ Methamphetamine Microplate ELISA for the detection of Methamphetamine in oral fluid

ABSTRACT

Abuse of methamphetamine is one of the major social problem faced by many countries. Oral fluid as an alternative matrix for assessing drugs of abuse is gaining prominence. It is therefore essential to investigate assay performance and limitations of screening techniques for methamphetamine in oral fluid. The purpose of this study was to evaluate the validity of Alere™ methamphetamine microplate competitive enzyme-linked immunosorbent assay (ELISA) for the analysis of methamphetamine in oral fluid. Ten samples were analysed in the laboratory using the Alere™ Methamphetamine ELISA kit, and the results were compared to the results obtained using gas chromatography-mass spectrometry(GC-MS) with good precisions (intra = 2.88%, inter = 9.04%) and accuracy (R^2 =0.9975). True negative, true positive, false negative and false positive results were determined by the GC-MS analysis. The result of the samples consisted 6 true negatives, 3 true positives and 1 false negative within the cut off concentration of 100 ng/mL. The results also demonstrated a functional sensitivity and specificity of 75% and 100% respectively. All the tested cross-reactive drugs showed cross-reactivity of less than 10% with methamphetamine except for MDMA which showed cross-reactivity of 44%. These data show that Alere™ methamphetamine microplate ELISA is a fast, precise and accurate screening technique for the detection of methamphetamine in oral fluid samples.

***Keywords*:** Methamphetamine; ELISA; Oral fluid; Cross reactivity

1. INTRODUCTION

Methamphetamine (METH) is a potent stimulant that affects the central nervous system. It was synthesised through the methylation of amphetamine, making it easy for permeation into the blood stream and brain [1-2]. METH, among other amphetamine derivatives, is the most widely abused drug because of its high potency. At comparable doses, a larger amount of it gets into the brain making it a more potent stimulant than others, including the parent amphetamine, thus making it a highly abused drug and hence causing peripheral sympathomimetic activity [3-4]. Therefore, a fast and accurate screening method of these drugs in biological matrices is of great importance.

39 Methamphetamine hydrochloride (crystal METH) which is the widely used form, exists as
40 white crystals or crystalline powder at room temperature with a bitter taste and has a melting
41 point between 170-175 ^{O}C and it is soluble in water and ethanol. METH decomposes on
42 heating, emitting toxic vapour of nitric oxides but stable under acidic and basic conditions
43 [5]. METH can be oxidized by human Flavin-Containing Monooxygenase Form 3 (FMO3) to
44 methamphetamine hydroxylamine which in turn can be oxidized to phenylpropanoid by
45 FMO3 [6].
46
47 According to 2013 national institute of drug abuse (NIDA) report series, METH can be
48 administered via injection, inhalation or oral ingestion and smoking depending on the forms,
49 with a slower occurrence effect from oral administration. Injection and smoking are the
50 common ways through which METH is administered, as these methods easily get the drug
51 into the brain and bloodstream, creating an instant drug's addiction potential as well as health
52 consequences [3].

53 The screening for METH abuse is said to be complicated, as analogues drugs such as
54 methylenedioxymethamphetamine (MDMA), methylenedioxyamphetamine (MDA),
55 methylenedioxymethamphetamine (MDEA) and D-amphetamine have been used by abusers
56 of METH to mimic its effects [7]. Kroener & Musshoff reported that most of these analogue
57 drugs are likely to test positive by some commercial immunoassay screening for METH [8].

58 The analysis of METH and its related drugs has recently involved the use of enzyme-linked
59 immunosorbent assay (ELISA) in most forensic laboratories [9-10]. This is due to its
60 adaptability for use with urine, oral fluid and blood samples without sample pre-treatment;
61 ease to use, low volume applicability and growing potential for automation [9]. ELISA relies
62 on the inherent ability of an antibody to bind to the specific structure of a molecule.

63 The use of oral fluid as an alternative matrix to blood and urine for the assessment of drug
64 status is on the increase. Oral fluid is readily available for collection and non-invasive with a
65 nominal chance of contamination when compared with blood and urine [11-12]. A similar
66 study reported a low accumulation of MDMA in plasma after administration of 75 mg of
67 MDMA than in oral fluid, with concentrations of 21-295 μg/L and 50-6982 μg/L for plasma
68 and oral fluid respectively [13]. Also, reviewed studies by de la Torre *et al*. on the clinical
69 pharmacokinetics showed a higher concentration of MDMA in oral fluid than in other
70 matrices [14]. Nevertheless, the administrative routs and collection procedure can greatly
71 affect the detection concentrations in oral fluid [10].

72 The purpose of this study was to evaluate the validity of Alere^{TM} METH microplate ELISA
73 as a screening method for the detection of METH in oral fluid samples since drugs detection
74 in biological matrices has legal implications. The results obtained using ELISA were
75 compared to a reference data collected from GC-MS. The accuracy of the assay was
76 determined, and the functional sensitivity and specificity of the test were calculated.

77

78 **2. MATERIALS AND METHODS**

2.1 Materials

2.1.1 Reagents and chemicals

Alere™ ELISA kit used for the METH screening contained the following; a ninety-six (96) well antibody coated micro strips, wash buffer solution of 0.1 %(v/v) surfactant, enzyme conjugated to horseradish peroxidase (HRP), substrate solution (3,3,'5,5'-tetramethylbenzidine) (TMB) and stopping solution (1.0 M sulphuric acid). 500 ng/mL MDMA, 500 ng/mL D-amphetamine, 500 ng/mL MDA and 500 ng/mL MDEA were used to test for cross-reactivity to METH. Four calibrators (standard solutions) (0, 25, 100 and 500 ng/mL) of METH in oral fluid were used. All the chemicals and reagents were of analytical grade and gotten from Sigma Aldrich, United Kingdom.

2.1.2 Apparatus

Microplate reader ELX800, multi-channelled pipette, an automated pipette, a Guardian centrifuge, sterile Eppendorf tubes (1.5 mL), sterile trough, Eppendorf rack and Fisher brand wash bottle were all used for this study.

2.1.3 Samples

10 oral fluid samples were obtained from the sample bank of Biosciences laboratory, Department of Biosciences and Chemistry, Sheffield Hallam University, UK. The samples were collected using Quantisal oral fluid collection device according to the manufacturer's instruction and were stored at -20 ^{o}C. The samples were tested and screened at Sheffield Hallam University, Bioscience laboratory for METH. The negative control sample was also obtained as above. The drug-free oral fluid sample was used as a negative control while a positive control sample of 100 ng/mL in oral fluid was prepared by the addition of 100 µL of working solution of METH at 1000 ng/mL to 900 µL of drug-free oral fluid.

2.2 Methods

2.2.1 Alere™ Methamphetamine Microplate ELISA

The ELISA screening used in this research is a competitive heterogeneous enzyme immunosorbent assay. The calibrators were placed in front and at the extreme of the 96 well plate, followed by the control samples, cross-reactive samples, the test samples and the linearity samples. The calibrators were analysed four times while the rest samples were analysed in duplicate. 25 µL each of the above-listed samples were pipetted into the 96 well plate. 100 µL of the enzyme conjugate was added to each of the wells, and the mixture was incubated for 30 minutes at ambient temperature. After the period of incubation was over, the wells were washed four times with 300 µL of the wash buffer to remove any unbound antigen sample. This was followed by the addition of 100 µL TMB substrate solution and the mixture was further incubated for 30 minutes at ambient temperature. This gave a varying degree of blue colouration depending on the concentration of methamphetamine in each well. Finally, the reaction was brought to a stop by adding 100 µL of the stop solution. The blue content of the wells turned yellow upon the addition of the stop solution. This yellow colouration

enables the multi-well plate reader to detect the chromophore at 450 nm, after which the absorption was measured at 450 nm within 30 minutes using ELX800 microplate ELISA reader. From the calibration curve obtained by plotting the calibrators concentrations against their respective absorbance, the corresponding methamphetamine concentrations were estimated [2].

2.3 Method validation

2.3.1 Accuracy and Precision

The accuracy of the assay was determined by comparing the measured ELISA results to the reference value obtained from GC-MS. Accuracy, as used here, is the closeness of agreement between the test results and the reference values [15].

The intra-assay (within a day) and inter-assay (between days for two weeks) precisions were calculated using the coefficient of variation (CV) from 10 replicate analyses of the 100 ng/mL positive control sample of the oral fluid.

2.3.2 Functional Sensitivity and Specificity

From the comparison of the ELISA results and GC-MS results, the true positive (TP), true negative (TN), false positive (FP) and false negative (FN) at the cut off concentration of 100 ng/mL were determined. The TP and FN rate was used to calculate the sensitivity of the assay using equation 1. While the TN and FP rate was used to determine the specificity of the assay using equation 2 [2,16].

$$\text{Sensitivity} = (TP \text{ x } 100/TP+FN) \quad 1$$

$$\text{Specificity} = (TN \text{ x } 100/TN+FP) \quad 2$$

2.3.3 Cross-Reactivity

The extent to which other drug substances cross-react with the immobilised antibody used for the analysis of METH was calculated by testing solutions of MDMA, D-amphetamine, MDA and MDEA in duplicate at 500 ng/mL concentrations. The percentage cross-reactivity was calculated by comparison of the measured concentrations with the actual levels of the cross reactants expressed in percentage [2,17].

2.3.4 Limit of detection (LOD)

The LOD of the assay was determined by measuring the negative control sample twenty times in a single assay. It was calculated from the mean absorbance value by applying equation 3, which yielded an absorbance value which was extrapolated from the calibrator's curve to give the LOD of the assay [2,18].

LOD = Ao – 2.5σ
3

Ao = Mean absorbance and σ = absorbance standard deviation

2.3.5 Linearity

Three concentrations of METH at 8.33 ng/mL, 16.67 ng/mL and 25 ng/mL were prepared by successive dilution of 25 µL, 50 µL and 75 µL of the stock solution (50 ng/mL) to 150 µL respectively with deionised water. The samples were analysed in duplicate to determine the linearity of the assay. The linear regression analysis is used to establish the relationship between the necessary response (y) and the analyte concentration (x).

2.4 GC-MS Analysis

The GC-MS used for this study was an Agilent 7890A with the 5975C run in electron impact ionisation mode, split-less injection and equipped with Restek Rtx®-5MS capillary column of 30 m, 0.25 mm and 0.25 µm. The injection port temperature was 250 ^{o}f with an injection volume of 1.0 µL. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The column temperature programme consisted of the initial temperature of 60 ^{o}C at 1 min which was ramped at 10 ^{o}C/min to 220 ^{o}C and then held for 4 min.

3. RESULTS AND DISCUSSION

The high level of abuse of METH in recent time has called for rapid growth in forensic and clinical analyses. It is therefore important to investigate immunoassay performance and limitations for drugs of abuse in different biological matrices.

The mean absorbance and percent coefficient of variation (%CV) of the calibrators provided by the assay are shown in Table 1.

In this study, a ready to use and reliable METH kit under routine laboratory conditions was used. This is due to the time-consuming optimisation of the calibrators to obtain the expected absorbance as indicated on the Alere^{TM} methamphetamine ELISA kit instructions. The generated absorbance was used to validate the assay for qualitative results as it is difficult to achieve reliable quantitative results with immunoassay [8]. Also, 1:5 dilution of the oral fluid samples in water was carried out to reduce background noise [2].

Table 1: Absorbance of calibrators in the Alere^{TM} methamphetamine ELISA kit

	Calibrator			
Conc (ng/mL)	0.00	25.00	100	500
Mean±SD	0.75±0.05	0.36±0.03	0.16±0.02	0.06±0.01
CV (%)	6.76	8.53	10.36	12.98

3.1 Accuracy and precisions

The ELISA results obtained for the ten test samples and the corresponding GC-MS results as reference standard were compared to ascertain the accuracy and validity of the assay as shown in Figure 1 and 2. From Figure 1, the linear regression of 0.9975 obtained from the graph by comparing both ELISA and GC-MS results showed that there is a close correlation between the two techniques. Also, the negative and positive control samples were confirmed negative and positive respectively by GC-MS at their cut off concentration.

The intro and inter assays precision of the Alere™ methamphetamine ELISA for ten replicates of positive control sample at 100 ng/mL METH was calculated from the estimated mean absorbance of 0.13±0.00 (2.88%) and 0.16±0.01 (9.04%) respectively. The intro and inter assay precision of the test samples were below 10% (Table 2).

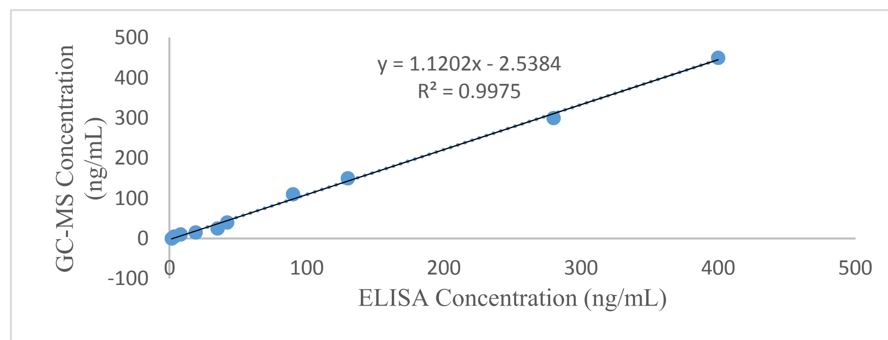


Figure 1: Comparative graph of GC-MS and ELISA

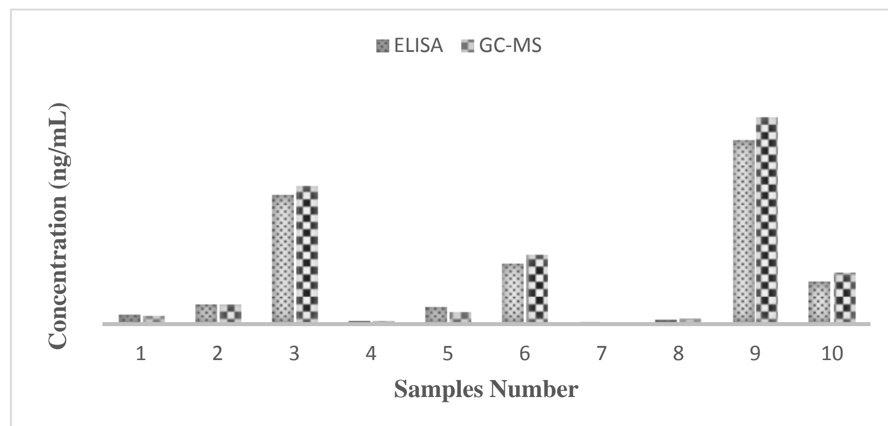


Figure 2: Analysis of METH concentration in Oral Fluid Samples by ELISA and GC-M

Table 2: Precision of positive control in the Alere™ methamphetamine ELISA kit

Precision	Mean±SD	CV(%)
Intra assay	0.13±0.00	2.88

Inter-assay	0.16±0.01	9.04

225
226

227 ### 3.2 Functional sensitivity and specificity

228 The sensitivity and specificity of the assay at a cut off concentration of 100 ng/mL is shown
229 in Table 3. Of the ten oral fluid samples tested, 60% were confirmed negative (the samples
230 produced both negative screening and confirmation results), 30% positive (they produced
231 both positive screening and confirmation results) and 10% was false negative (the sample
232 produced negative screening and positive confirmation result) within the cut off
233 concentration of 100 ng/mL. The sensitivity and specificity are necessary for validation, as
234 they provide insight into the ability of the assay to categorize samples as negative or positive.
235 The sensitivity obtained was 75%, and the specificity was 100%. The specificity obtained
236 was excellent, but the limitation in sensitivity at the cut off concentration was due to the false
237 result produced in this study.

238

239 Table 3: Sensitivity and specificity of the Alere^{TM} methamphetamine ELISA kit

S/N	ELISA (ng/mL)	GC–MS (ng/mL)	Result
1	19	15	TN
2	42	40	TN
3	280	300	TP
4	3.5	5	TN
5	35	25	TN
6	130	150	TP
7	1.6	00	TN
8	8	10	TN
9	400	450	TP
10	90	110	FN
Sensitivity (%)	75		
Specificity (%)	100		

240 TP = True Positive, FP = False Positive, TN = True Negative, FN = False Negative

241

242 ### 3.3 Cross-Reactivity

243 The cross reactivities for the Alere^{TM} methamphetamine microplate ELISA screening is given
244 in Table 4. All the drugs tested showed zero or little cross reactivities of less than 10% with
245 METH with an exception of MDMA which showed cross-reactivity of 44%. This is in line

246 with the Cozart® methamphetamine microplate ELISA having approximately 50% cross-
247 reactivity with MDMA [9]. This could be due to the ability of the antibody immobilised on
248 the microplate to recognise MDMA having structural molecule similar to METH molecule
249 [17]. These discoveries were important to distinguish between METH and other closely
250 related drugs as; these drugs are capable of producing false positive results in the reflection of
251 METH. Although ELISA technique could be considered as being specific to the analyte of
252 interest(METH). However, there was a significant cross-reactivity with MDMA drug.
253 Therefore, it is important to have a screening that is as specific as possible for METH.
254

255 Table 4: Relative cross reactivities of Alere™ methamphetamine ELISA with
256 methamphetamine

Drug Conc. (ng/mL)	Compound	Measured Conc. (ng/mL)	Percent cross-reactivity (%)
500	MDMA	220	44
500	D-Amphetamine	ND	ND
500	MDA	38	7.6
500	MDEA	1.3	0.26

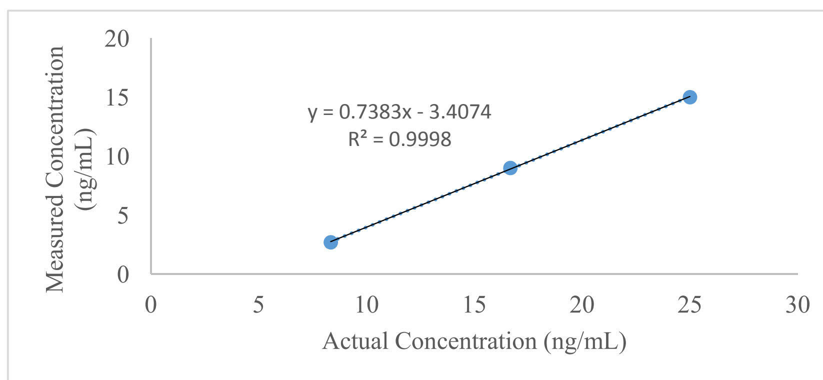
257

258

259 **3.4 Limit of detection (LOD) and Linearity**

260 The LOD of the assay was calculated to be 1.6 ng/mL, and the mean absorbance of the
261 twenty replicates of the negative control sample was 0.74±0.02 standard deviation.

262 The Alere™ methamphetamine microplate ELISA assay shows good linearity with
263 regression coefficient (R^2) of 0.9998 as shown in Figure 2.



264

265 Figure 3: Linear graph correlating measured and actual concentration of METH using ELISA
266 kit

267

268 ## 4. CONCLUSION

269 A screening technique for the detection of methamphetamine in oral fluid has been validated.
270 In general, the results show that the Alere^{TM} methamphetamine microplate ELISA is specific,
271 rapid and accurate for screening METH positive oral fluid sample. However, there was a
272 significant cross-reactivity with MDMA drug. Cross-reactivity tendencies in ELISA
273 technique could be regarded as a major setback since results obtained have to be further
274 confirmed using a more specific technique like GC-MS. Therefore, ELISA technique should
275 be validated for each type of drugs in different matrices.

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