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Methadone Extraction using Solid Phase Extraction from Urine and Quantification by Gas Chromatography-Mass Spectrometry

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Abstract:- It is a vital requirement to improve analytical methods to identify individuals of drugs of abuse. Analytical method was developed to identify methadone in urine specimens using solid phase extraction (SPE) and gas chromatography-mass spectrometry (GC-MS). Gracepure™ C18 column was used for SPE and GC-MS was operated in electron impact ionization full scan mode. Hexadecane was used as an internal standard. The SPE-GC-MS method was validated for urine spiked with methadone with a concentration range of 0.010 to 0.050 mg mL^{-1} and acceptable linearity (R^2 = 0.9905 ± 0.0052) was observed. The LOD and LOQ values for urine spiked with methadone obtained in this research were 0.193 ± 0.008 µg mL^{-1} and 0.607 ± 0.023 µg mL^{-1} respectively. The developed method was convenient for the extraction and quantification of methadone in urine.

Keywords:- *Methadone, Hexadecane, Gas Chromatography-Mass Spectrometry, Solid Phase Extraction, C18 Column.*

I. INTRODUCTION

Analysis of biological matrices is usually carried out for work place drug testing programs [1], roadside testing [1], sports competitions [2], medical treatment purposes [3], criminal investigations and monitoring the compliance of drug maintenance programs [2]. On-site tests and laboratory tests are two kinds of tests currently utilise for analysing urine in different social groups of drugs of abuse [4]. Various analytical techniques including the immunoassay principle and the instrumental technology have been utilised for detection of individuals of drugs of abuse [5] and therefore, today isolation and analysis of drugs have become routine work in the most of the forensic science laboratories as a confirmation of individuals of drugs of abuse.

A large number of drugs have been currently classified as illicit or controlled substances [6]. Global economy and information technology are dramatically affected to spread illicit substances all over the world [7]. It is a vital requirement to improve analytical methods to identify persons involved in drugs of abuse. Consequently, in this study gas chromatography-mass spectrometry (GC-MS) is utilised as an instrumental method for detection of synthetic drug, methadone after extraction from urine using solid phase extraction (SPE).

A. Methadone

Methadone, chemically known as 6-dimethylamino-4,4-diphenyl-3-heptanone (Amidine, Symoron, Dolophine and more other names) is a synthetic opioid used to treat pain was developed in Germany around 1939 by Gustav Ehrgart and Max Bockmuhl [8]. This synthetic opioid is described as an analgesics and very useful therapeutic for long term relapsing heroin addicts [9]. Cheng *et al.* (2008) [10] mentioned that methadone causes respiratory depression, stupor, hypotension, and circulatory problems.

Methadone is categorised as a class A substance controlled under schedule 2 of the Misuse of Drugs Act 1971 [11]. Methadone metabolism occurs in the liver [12] and it is metabolised by mono- and di-N-demethylation to form 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) an inactive metabolite and 2-ethyl-5-methyl-3,3-diphenylpyrroline (EMDP) [10]. The cytochrome P450 isoform CYP3A4 is responsible for the metabolism of methadone to EDDP [13].

Alburges*et al.* (1996) [9] carried out a research to identify methadone, EDDP and EMDP in body fluids of urine, plasma and liver microsomes and found the limit of quantification (LOQ) as 10 ng mL^{-1} for all three analytes.

B. Urine as Biological Matrix

Urine is widely accepted body fluid for detection of drugs of abuse testing because it is an inexpensive method, high accuracy level between 97% and 98% [14] and contains a high concentration of drugs [5]. Analysis of various types of drug in urine is economical, reliable, regulated and extensively employed both in the workplace and wider community [15]. Urine contains greater than 95% water and urea, chloride, sodium, potassium ions cover the balance with creatinine. The components and their percentages which effect to the analysis of the analyte of interest can be vary in urine mainly due to liquid intake and meals [16].

Hall *et al.* [17] indicated that comparison to plasma, urine has much lower protein content and fewer matrix components but pH of urine is variable compare to the plasma and therefore pH adjustment of samples may be required. The detection period of drugs and their metabolites in urine is usually from 1 to 3 days [18].

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C. Overcoming Matrix Effects

The analysis of biological specimens to determine the accurate concentration of drugs and its metabolites require proper sample preparation [19]. Addition of phosphate buffer for pH adjustment is suggested by Lillsunde and Kortefor urine [20]. Sometimes urine samples contain particulate matter and it is advisable to filter or centrifuge the samples before analysis [21].

Several other methods have also been previously identified and these methods are important to prepare a quality sample prior to analysis of drugs and the methods used in sample preparation are SPE [22], supported liquid membrane extraction [23], liquid-liquid extraction [24], solid phase micro extraction [25, 26] and headspace extraction [27].

Scheurer and Moore (1992) [28] reviewed that trend moves towards the SPE for analysis of biological matrices due to less solvent consumption, reduced time of operation, higher extraction efficiency and also answer for a minimum quantity of toxicological specimens. In addition to that automation of SPE process achieved some other advantages comparatively less manual work and minimize the risk of human errors [19].

D. Solid Phase Extraction

SPE is a sample preparation method which concentrates and purifies analytes from appropriate solution by adsorption onto a various modified chemical substances [21]. Simpson NJK (2000) [29] reported that initially the term 'solid phase extraction' was introduced by Zie*fet al.* in 1982. Generally isolation and concentration of drugs from body fluids using SPE of silica-based packing involves four steps: conditioning of sorbent bed, application of the biological sample, washing the sorbent bed and elution of the drug of interest [30].SPE methods have been successfully applied to extract several drugs and their metabolites from biological matrix of urine [31].

Common sorbents used to SPE process are reversed phase column, normal phase column, ion exchange and size exclusion [21]. Octadecyl (C-18), octyl (C-8), cyclohexyl and phenyl-functional groups chemically bonded to silica are examples for reversed phase columns [21]. Reversed phase columns contain more hydrophobic packing material than the sample used to analysis [21]. This type of columns adsorb the presence impurities and interferences in the sample and therefore have generally been utilised for extraction of the drug prior to analysis [21]. Reversed phase column containing nonpolar C-18 is an ideal for use with aqueous specimens over a wide range of polarities [21].

The primary purpose of this study is to develop an efficient method for extract methadone from urine utilising Grace Pure™ C18 column and GC-MS. Initially a suitable temperature program, injection volume, flow rate and column will be selected for GC-MS to analyse the samples. Moreover substances will be identified using retention times.

SPE process will be developed to maximize extraction of methadone from urine for quantification purpose. It will be carried out economically by utilising ultrapure water and minimum amount of organic solvents such as methanol and hexadecane.

The developed SPE method will be validated in accordance with the standard validation parameters of United Nations Office on Drugs and Crime (UNODC) [32]. Linearity, LOD and LOQ will be assessed as standard validation parameters.

II. METHODOLOGY

A. Chemicals and Instrumentation

Reference standards of methadone hydrochloride was purchased from Macfarlan Smith (Edinburgh, UK). Hexadecane and methanol HPLC grade 99.9% were purchased from Sigma Aldrich® (UK). All the chemicals and reagents were used without modification unless otherwise stated.

Ultrapure water used for column conditioning and washing stages was produced using Barnstead™ Easypure™ RoDi water purification system (Triple Red Limited, Buckinghamshire, UK).Fresh urine samples utilised for analysis were collected from a drug free healthy volunteer.

Analytical digital balance, Precisa XB 120A (Milton Keynes, UK) was used for all weight determinations. For calibration standard and sample preparation borosilicate glassware was used. Volac® disposable glass Pasteur pipettes (230 mm) and 50 µL micro syringe (Hamilton, USA) were also used for calibration standard and sample preparation. The SPE columns GracePure™ C18 - low (100 mg mL^{-1}) were obtained from Alltech Associates, (Carnforth, UK). A Jouan® centrifuge, Whirlimixer vortex mixer and solid phase extraction manifold coupled with piston vacuum pump FB65540 (Fisher Scientific, UK) were used for sample preparation. For sample concentration purpose Techne sample concentrator was acquired from Bibby Scientific Limited, UK. 2 mL glass GC vials capped lids used for calibration standard and sample analysis were purchased from Agilent Technologies® (California, USA).

An Agilent 6850 gas chromatograph equipped with an Agilent 6850 series auto sampler, coupled to an Agilent 5975C mass selective detector was used for all chromatographic analyses (California, USA). The GC column used to separate individual components was 30.0 m x 250 µm x 0.25 µm HP-5MS 5% phenyl methyl siloxane capillary column supplied by Agilent Technologies® (California, USA). Chromatographic data analysis was performed using MSD Chemstation software G1701EA E.02.00.493 (California, USA). All the statistical analysis was carried out using Excel software, version 2013.

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B. Preparation of Standards and Samples

Methadone stock solution was prepared in a concentration of 2.0 mg mL^{-1}, free base using methanol. Hexadecane solution of 2.0 mg mL^{-1} was also prepared in methanol to use as an internal standard.

C. Spiked urine samples

Analysis of methadone in urine was carried out after preparation of solutions as follows. Working solutions of methadone were prepared in concentrations of 0.20,0.30, 0.40, 0.60 and 1.0 mg mL^{-1}. Fifty microlitres of each working solution was diluted with 950 microlitres (0.95 mL) of fresh urine and this gave solution concentrations of 0.010, 0.015, 0.020, 0.030 and 0.050 mg mL^{-1} calibration standards.

The first stage of experiment 0.80 mg mL^{-1} internal standard hexadecane was included in the above working solutions to result in 0.040 mg mL^{-1} of hexadecane in each urine calibration standard. Later 0.040 mg mL^{-1} solution of the internal standard was prepared separately to reconstitute the methadone samples.

D. Method Development

Prior to analyse spiked urine samples for methadone, GC-MS method was developed by employing previously published two methods by Goldberger *et al*. (1993) [33] and Moeller *et al*. (1993) [34] for analysing opiate drugs. This method development was successfully carried out by using the solution of concentration of 0.010 mg mL^{-1} methadone containing 0.040 mg mL^{-1} hexadecane internal standard to compare the above two methods utilising GC-MS.

E. Urine Extraction Procedure

Blank urine sample and methadone spiked urine samples (0.010, 0.015, 0.020, 0.030 and 0.050 mg mL^{-1}) were centrifuged at 3000 revolutions per minute (rpm) for 10 min. Liquid components of the samples were loaded on to the SPE column using disposable glass Pasteur pipettes while pellets were discarded.

The extraction procedure consists with four steps. Initially, conditioning of the solid phase sorbent beds were done by adding 2 mL of methanol followed by 2 mL of ultrapure water. Next, blank urine sample and spiked urine samples were added to the respective columns. At the third step, each column was washed with 4 mL of ultrapure water followed by applying full vacuum for 3 min. At the elution step analytes of interest were collected by using 3.0 mL of methanol.

Samples and solvents used in the extraction process were enabled to pass slowly under the gravity pressure. SPE columns were not allowed to dry at the conditioning stage and the sample adding stage.

The purified extracts were evaporated to dryness under the stream of nitrogen at 40 ℃. Dried extracts were reconstituted with 0.50 mL of 0.040 mg mL^{-1} internal standard solution and transferred to glass GC auto sampler vials.

F. Instrumental Procedure

All standards and spiked urine samples were analysed in triplicate in a consecutive sequence. Methanol blank was run at the beginning and at the end of each consecutive sequence and after each calibration standard. The sequence was set as urine blank at the beginning and thereafter the lowest to the highest concentrations of 0.010, 0.015, 0.020, 0.030 and 0.050 mg mL^{-1} calibration standards of spiked urine samples.

The GC-MS was operated in the splitless mode with the injection volume of 3.0 µL. Helium carrier gas was used with a pressure of 16.00 psi and a total flow rate of 1.2 mL min^{-1}. Four pre injection methanol washes, three pre injection sample washes and six post injection methanol washes were carried out. The injection port temperature was set at 250 ℃. The oven temperature was programmed as follows. Initial oven temperature 150 ℃ held for 1 minute; increased at 12.5 ℃ min^{-1} to 200 ℃; held for 15 sec; and again increased at 30 ℃ min^{-1} to 290 ℃ held for 2 min; giving a total running time 10 min 15 sec.

The temperatures of the quadrupole, ion source and mass selective detector interface were 150 ℃, 230 ℃ and 285 ℃, respectively. The electron impact ionization mode of 70 eV was used for the ionization of the analytes.

G. Method Validation

- *Linearity*

The linearity of the developed solid phase extraction method was determined in the triplicate analysis of each spiked urine standard of methadone (0.010, 0.015, 0.020, 0.030, 0.050 mg mL^{-1}). Linearity was determined by plotting the peak area ratio (peak area of methadone spiked urine calibration standard over the peak area of internalstandard hexadecane) obtained from methadone and internal standard versus methadone concentration. The linear regression line was calculated by utilising the least squares regression method. The linear regression line and the coefficient of determination (R^2) were used to plot the calibration curve. The R^2 value higher than 0.99 was considered as an acceptable linearity over the working range of concentrations of the analyte of interest [32].

- *Limit of Detection (LOD) and Limit of Quantification (LOQ)*

The LOD and LOQ were determined by analysing urine blank and methadone spiked urine standards in 0.10, 0.25, 0.40, 0.70 and 1.0 µg mL^{-1} concentration. Standards were prepared by diluting fifty microlitres of 2.0, 5.0, 8.0, 14.0, 20.0 µg mL^{-1} concentration working solutions with 950 microlitres (0.95 mL) fresh urine. Above series of concentration was decided according to the LOD value obtained by Paterson *et al*. (2000) [35].

To obtain the LOD and LOQ values data was gathered by manually measuring the peak (signal) height of methadone spiked urine standards of triplicate runs and the peak height of triplicate runs of the urine blank (baseline).

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The LOD and LOQ for spiked urine specimens were determined according to the guidelines of UNODC making series of dilutions to establish the concentration at a signal to noise (S/N) ratio of 3:1 and 10:1 respectively [32].

III. RESULTS AND DISCUSSION

Previously published two methods by Goldberger *et al*. (1993) [33] and Moeller *et al*. (1993) [34] were programmed separately and concentration of 0.010 mg mL^{-1} methadone with 0.040 mg mL^{-1} hexadecane solution was analysed using GC-MS. According to the both methods 5% phenyl methyl siloxane capillary column was used for opiate drugs analysis in the splitless mode of injection. However temperature program of Moeller *et al*. [34] started from 70 ℃ (1 min hold); increased at 30 ℃ min^{-1} to 155 ℃; increased at 10 ℃ min^{-1} to 240 ℃; and again increased at 30 ℃ min^{-1} to 300 ℃ (5 min hold). Total running time was 19 min and 20 sec. The heliumcarrier gas flow rate was 2 mL min^{-1}. Sample injection volume was 1.0 µL. Goldberger *et al*. (1993) [33] method was programmed in GC-MS, according to the detailsdescribed previously in instrumental procedure. The typical GC-MS chromatograms obtained from above two methods are shown in figure 1 and figure 2. According to the chromatograms, higher intensity peaks were obtained for hexadecane and methadone by the method of Goldberger *et al*. (1993) [33] compared to the method of Moeller *et al*. (1993) [34]. Moreover it indicated that retention time for the same analytes were varied with the GC-MS method i.e. temperature program and flow rate of the carrier gas used in the GC-MS.

It is absolutely true that method used by Goldberger *et al*. (1993) [33] was applied in this research to determine methadone due to its higher peak intensity. According to the values in figure 1 and 2,hexadecane and methadone have retention times of 2.269 minutes and 6.156 minutes respectively.

A. Solid Phase Extraction

In general, nonpolar octadecyl silica separates analytes from polar mobile phase due to nonpolar interactions of the sorbent bed and it can be eluted using nonpolar solvent by disrupting the nonpolar interactions [21]. In this research, methadone was isolated from urine using the C18 column as the sorbent bed and methanol as the solvent.

Water has the highest polarity index 10.2 and the respective value for the methanol is 6.6 [21]. It indicates that compared to water, less polar methanol is a suitable solvent to remove analyte of interest from the C18 column and therefore, analytes were collected by disrupting the interactions using methanol as an organic solvent. However according to the analysis results calibration curve was not acquired for 0.50 mL of solvent methanol. The reason for this was all the analytes were not properly eluted to the solvent.

The several possibilities can be caused for this issue. The presence of any water molecules on the sorbent bed can be reduced the elution efficiency by minimizing the contact between sorbent bed and methanol. Therefore it is vital to remove all water molecules in the column by applying vacuum pressure before adding methanol to elute the analytes.

It is important to apply the gravity pressure at the all four stages of SPE; conditioning the column, application of the urine sample, washing the sorbent bed and eluting of the analyte with solvent. Applying vacuum pressure to the column is caused to lower the extraction of the analyte. But vacuum pressure has to be applied before adding methanol to prevent the interruptions caused by remaining water molecules in the sorbent bed and also it must be applied finally to collect the elution solvent completely from the column followed by gravity pressure.

Moreover, it can be caused due to the insufficient volume of solvent to elute all the analytes of interest in the sorbent bed. Accordingly, in this study the effect of different solvent volumes for elution by carrying out fraction collection was further investigated.

B. Maximum recovery of Analytes

In this research, the minimum required volume of solvent methanol to maximize the recovery of methadone and hexadecane in the standards was also assessed.

Therefore, analysis was carried out by collecting six portions of 0.50 mL of elution solvent at concentration of 0.050 mg mL^{-1} methadone with 0.040 mg mL^{-1} hexadecane as an internal standard in urine. Each portion was collected separately under the gravity followed by applying full vacuum for 30 sec.

Usually, samples were sequenced from the lowest concentration to the highest concentration to reduce the risk of column priming. Because of the interaction between the compounds and the column path way, a priming effect which influence to the analysis may be noticed. By assuming elution stage 6 contained the lowest concentration compared to the other elution stages sequence was selected from elution stage 6 to elution stage 1 to obtain raw chromatograms. These chromatograms are shown figure 3 to figure 8 and the summary of the chromatograms obtained from GC-MS is given in Table 1.

With the initial 0.50 mL of methanol, there was a maximum recovery of hexadecane, however there was a very low intense peak for methadone without a considerable recovery. Integration of this low intense methadone peak was impossible and therefore no data in Table 1 for elution stage 1 for methadone peak area due to its poor recovery.

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Table 2: Table of data showing urine spiked with methadone standards (0.010 to 0.050 mg mL^{-1}) used for method validation in the linearity assessment. Showing methadone peak area (AM), internal standard hexadecane peak area (AIS) and peak area ratio (AM/AIS) (n=3)

[Methadone]	AM[a] (n=3)	AIS[b] (n=3)	AM/AIS[c] (n=3)	RSD[e]
mg mL^{-1}	Average ± SD[d]	Average ± SD[d]	Average ± SD[d]	(%)
0.010	1578061 ± 158760	9686282 ± 1121749	0.1632 ± 0.0055	3.342
0.015	3055437 ± 256418	10664893 ± 438052	0.2862 ± 0.0132	4.625
0.020	5077014 ± 269535	10910287 ± 410873	0.4663 ± 0.0404	8.673
0.030	12055476 ± 518996	10688118 ± 667975	1.1302 ± 0.0715	6.326
0.050	24200327 ± 538330	11275058 ± 463476	2.1476 ± 0.0518	2.412

[a] Methadone peak area
[b] Internal standard hexadecane peak area
[c] Peak area ratio (methadone peak area / internal standard peak area)
[d] Standard deviation (n = 3)
[e] Relative standard deviation (n=3)

Calibration curve for the determination of linearity of urine spiked with methadone standards is shown in Figure 11.

The developed method can be described "*as linear when there is a directly proportional relationship between the response and the concentration of analyte in the matrix over the range of analyte concentration of interest*" [32]. The coefficient of determination (R^2) is a statistical value and 0.99 is often used as the criterion of linearity. The R^2 value over 0.99 indicates good fit usable calibration curve and suitability for application in the quantitative analysis. Nevertheless, UNODC guidelines mentioned that methods with a coefficient of determination of less than 0.99 are also fit for purpose [32]. The average R^2 value for methadone obtained in this research was 0.9905 ± 0.0052 (n=3). This indicates that a good linear relationship exists between average peak area ratios over the methadone concentration range of 0.010 to 0.050 mg mL^{-1}. Therefore this working range is acceptable for analysis of methadone in urine taken from individuals of drugs of abuse.

It is absolutely true that the methanol blank has to be used to find out any possible carry-overs which can affect to the baseline and ultimately to the quantification. However, there were no any carry-overs were observed in methanol blank at each corresponding injection.

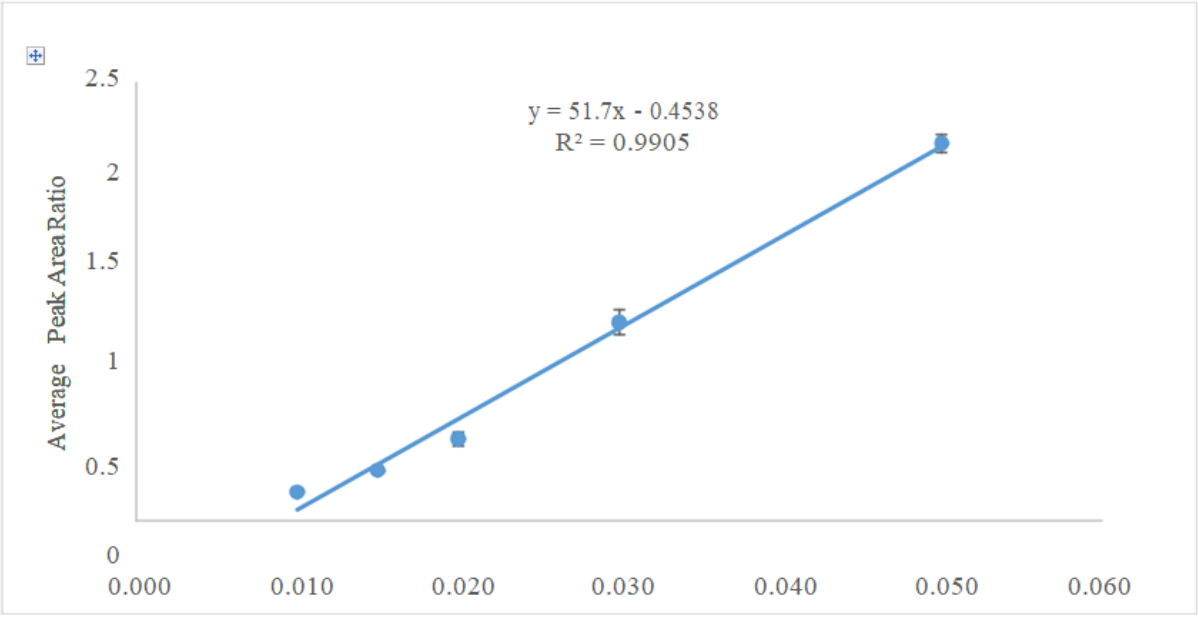

Fig. 11: Calibration curve of urine spiked with methadone standards used in method validation in the assessment of linearity. Showing the average peak area ratio (peak area of methadone/ peak area of internal standard) against the concentration of methadone from 0.010 to 0.050 mg mL-1 (n=3) obtained from analysis in GC-MS using electron impact ionization full scan mode and a flow rate of 1.2 mL min-1. Error bars representing standard deviations (SD) of the average peak area ratios are included but some points are not visible due to the lower error values compared to the scale of the y-axis.

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C. Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD is defined as "*the smallest measured content from which it is possible to deduce the presence of the analyte with reasonable statistical certainty*" [32].Moreover, the LOD is referring to the lowest analyte concentration that the analytical procedure can reliably differentiate from background noise [32]. It is absolutely true that reliability of drug detection declined close to the LOD value [4]. The presentation of the relevant chromatograms is considered acceptable for justification [37] becausethe LOD is not a robust parameter and can be affected by minor changes in the analytical system such as purity of reagents and matrix effects [32].

The LOQ is defined as "*the smallest measured content from which it is possible to quantify the analyte with an acceptable level of accuracy and precision*" [32]. Inforensic drug analysis experimentally determined LOD and LOQ values utilise signal to baseline (noise) ratios of 3:1 and 10:1 respectively [4, 32, 37].

The results obtained from the urine blank and urine spiked with methadone standards used to construct calibration curve for LOD and LOQ values determination are summarized in Table 3.

Table 3: Table of data showing urine spiked with standards of methadone (0.10 to 1.0 µg mL^{-1}) used for method validation to determine the LOD and LOQ. Showing methadone peak height (HM), urine blank baseline peak height (HU) and signal to noise ratio (HM/HU) (n=3)

[Methadone]	**HM[a] (n=3)**		**HU[b] (n=3)**	**HM/HU[c] (n=3)**	**RSD[e]**
µg mL^{-1}	**Average ± SD[d]**		**Average ± SD**	**Average ± SD**	**(%)**
0.10	1233	± 57.74	550 ± 50	2.24 ± 0.105	4.68
0.25	1867	± 115.47	550 ± 50	3.39 ± 0.210	6.19
0.40	3900	± 264.58	550 ± 50	7.09 ± 0.481	6.78
0.70	4683	± 28.87	550 ± 50	8.52 ± 0.052	0.62
1.0	10233 ± 230.94		550 ± 50	18.61 ± 0.420	2.26

[a] Methadone (signal) peak height
[b] Urine blank baseline peak height
[c] Signal to noise ratio (signal / baseline)
[d] Standard deviation (n=3)
[e] Relative standard deviation (n=3)

Calibration curve for the determination of LOD and LOQ of urine spiked with methadone standards is shown in Figure 12. LOD and LOQ were determined by using linear regression equation of y = 16.902x – 0.2601. The R^2 value obtained to determine the LOD and LOQ was 0.9339 ± 0.0088 and it indicated that this value does not meet the criteria for the linear relationship between average signals to baseline ratio for the concentration of methadone up to 1.0 µg mL^{-1}. It can be noted that the reason to obtain lower R^2 value was that methadone concentration of 0.70 µg mL^{-1} was not achieved to the average signal to baseline ratio of regression line due to the error in solution preparation or extraction process.

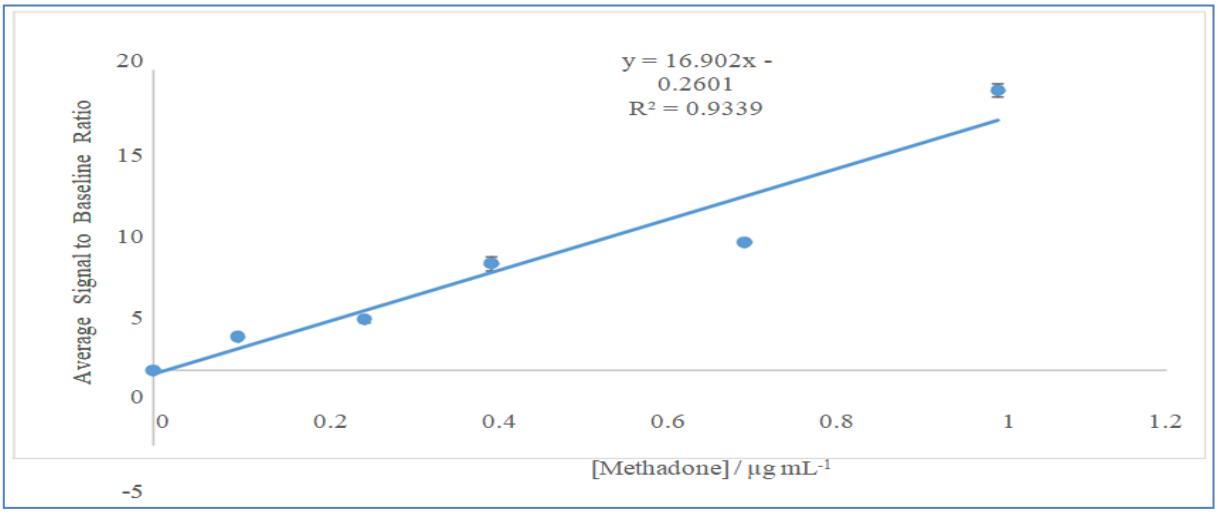

Fig. 12: Calibration curve of spiked urine with methadone standards used in method validation of LOD and LOQ determination. Showing the average signal to baseline ratio (peak height of methadone/ peak height of urine blank) against the concentration of methadone from 0 to 1.0 µg mL^{-1} (n=3) obtained from analysis in GC-MS using electron impact ionization full scan mode and a flow rate of 1.2 mL min^{-1}. Error bars representing standard deviations (SD) of the average signal to baseline ratios are included but some points are not visible due to the lower error values compared to the scale of the y-axis.

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The LOD value of urine spiked with methadone standards was calculated at signal to baseline ratio 3:1 and the value obtained in this research was 0.193 ± 0.008 µg mL^{-1}. The LOQ of urine spiked with methadone standards was calculated at signal to baseline ratio 10:1 and the value obtained as 0.607 ± 0.023 µg mL^{-1}. Paterson *et al*. (2000) [35] studied the detection limit of several drugs spiked in urine using GC-MS and obtained the detection limit for methadone was 0.2 µg mL^{-1}. The SPE processwas carried out by using C18 and cationic mixed mode adsorbent. However, value for the LOQ was not analysed by Paterson *et al* [35]. It showed that the LOD value obtained from this research is almost similar to the value obtained by Paterson *et al*. in 2000 [35].

The LOD and LOQ values obtained for methadone in this research are compared with the values published for urinalysis using other GC methods by Alburges*et al*. (1996) [9], Cheng *et al*. (2008) [10] and Cheong *et al*. (2010) [38]. Alburges*et al*. (1996) [9] developed a GC-PICI-MS method for the determination of methadone and its metabolites using C8 and cation exchange column. Mass spectrometry was operated in the positive ion detection mode and data were collected using SIM mode. The reported LOD and LOQ of methadone were 5 and 10 ng mL^{-1} respectively.

In the year 2008, Cheng *et al*. [10] carried out a comprehensive study for simultaneous determination of several drugs including methadone in urine using GC-MS in the SIM mode for quantification. Sample clean-up process was carried out by applying SPEC DAU SPE (3 mL) cartridges. The LOD and LOQ values obtained for methadone were 5 and 20 ng mL^{-1} respectively. Moreover, Cheong *et al*. (2010) [38] showed an analysis of urine for methadone and other illicit drugs and acquired 10 ng mL^{-1} of LOD value for methadone using GC-MS in the SIM mode for screening and quantification.

The LOD and LOQ values obtained for methadone in this research were higher than those values obtained by Alburges*et al*. (1996) [9] Cheng *et al*. (2008) [10] and Cheong *et al*. (2010) [38]. When using "…*full mass scan mode … the ions of each m/z only spend a very short time in the analyser, hence, only a small fraction actually reach to the detector*" [6]. However at the SIM mode ions of the targeted m/z aredetected by enhancing sensitivity. Accordingly, lower LOD and LOQ values were obtained by above three studies.

Working range is defined as "*the interval over which the method provides results with an acceptable uncertainty*" [36]. The lower value of the working range is determined by the LOQ level and selected working range (0.010 to 0.050 mg mL^{-1}) in this research can be further declined until 0.607 ± 0.023 µg mL^{-1}, the LOQ value of this research. Sometimes the upper value of the working range will be limited according to the instrument used for analysis [36] such as GC-MS due to its plateauing effect of very high concentration values.

IV. CONCLUSION

In this research, a simple method was developed for isolating and quantification of methadone in urine samples utilising C18 SPE columns. The analysis was performed by using GC-MS. It is noticeable that different analytes have different retention times and also retention time of the same analyte is changed according to the GC-MS method applied.

Analysis of six portions of 0.50 mL of methadone eluate at elution stage 1 to 6 demonstrated that most of the methadone can be recovered using the total volume of 3.0 mL methanol as the elution solvent. Method validation was carried out according to the standard validation parameters mentioned by UNODC [32]. Linearity, LOD and LOQ values were the validation parameters used to assess this extraction method. Validation of this C18 extraction method followed by GC-MS for the analysis of methadone in urine matrix indicated that it was a convenient method for drug analysis. Calibration curve acquired from urine spiked with standards of methadone was linear over the concentration range of 0.010 mg mL^{-1} to 0.050 mg mL^{-1}, with a R^2 value of 0.9905 ± 0.0052 (n=3). The LOD value obtained for this method was 0.193 ± 0.008 µg mL^{-1} which was close to the value achieved by Paterson *et al*. (2000) [35]. The LOQ value was read as 0.607 ± 0.023 µg mL^{-1}.

Though SPE consumes more time for methadone extraction, chromatographic running time of GC-MS was 10 min and 15 sec in this research.

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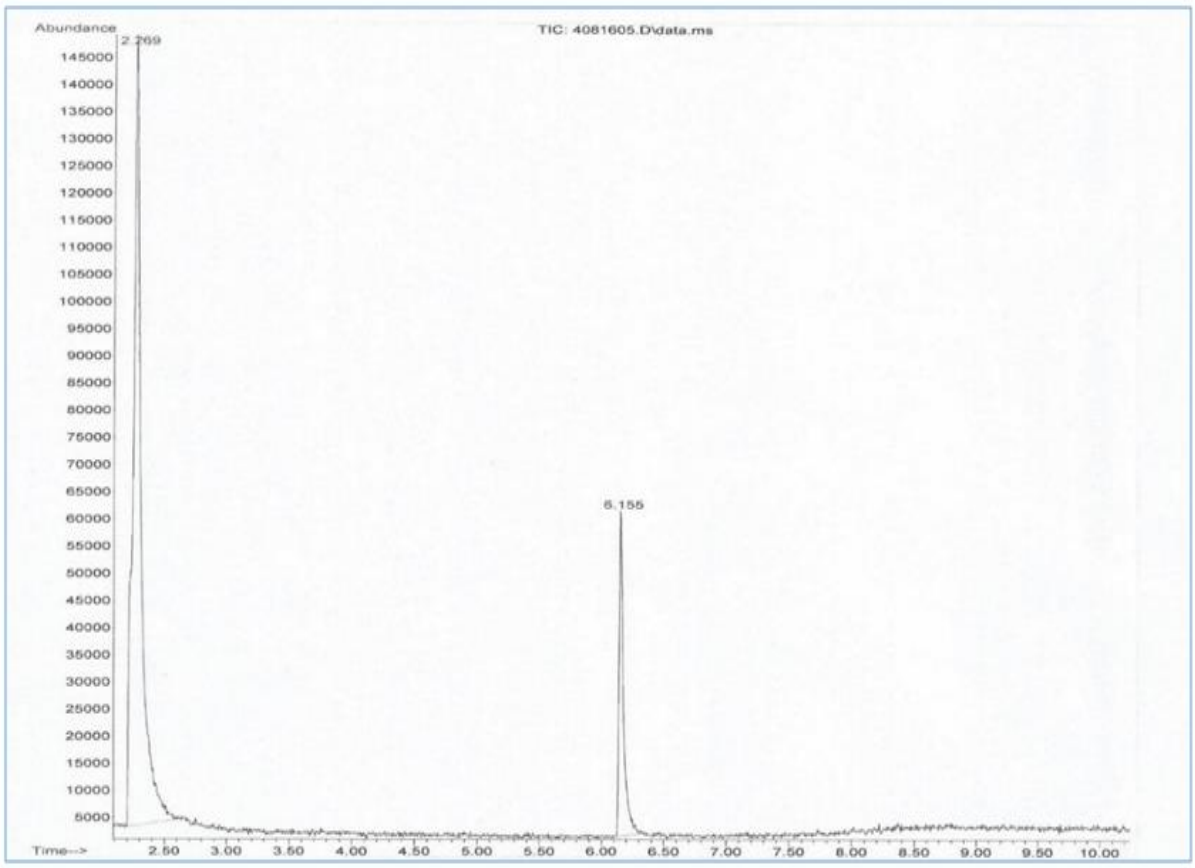

Fig. 1: Typical GC-MS chromatogram of concentration of 0.010 mg mL^{-1} methadone with 0.040 mg mL^{-1} hexadecane as an internal standard used to optimisation GC-MS method for methadone analysis showing the abundance (counts) against time (min) for method of Goldberger *et al*.

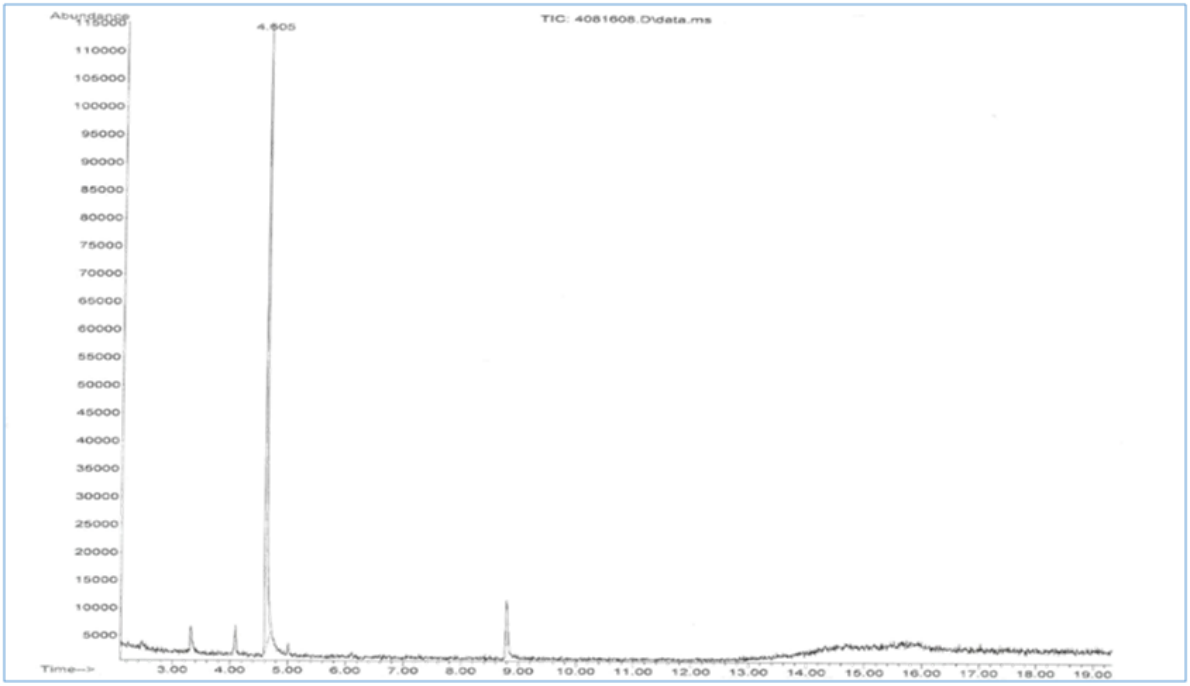

Fig. 2: Typical GC-MS chromatogram of concentration of 0.010 mg mL^{-1} methadone with 0.040 mg mL^{-1} hexadecane as an internal standard used to optimisation GC-MS method for methadone analysis showing the abundance (counts) against time (min) for method of Moeller *et al*.

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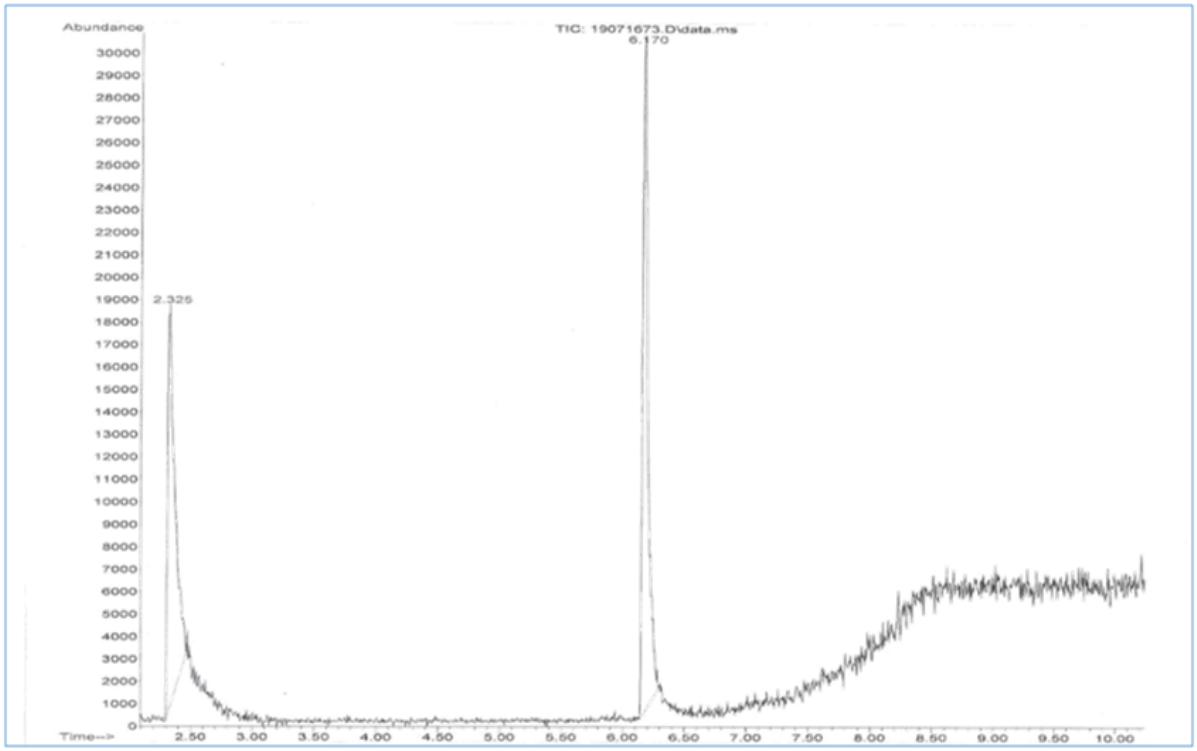

Fig. 3: Typical GC-MS chromatogram used to assess the volume of methanol required to recovery concentration of 0.050 mg mL^{-1} methadone with 0.040 mg mL^{-1} hexadecane as an internal standard showing the abundance (counts) against time (min) at elution stage 1 for 0.50 mL of methanol.

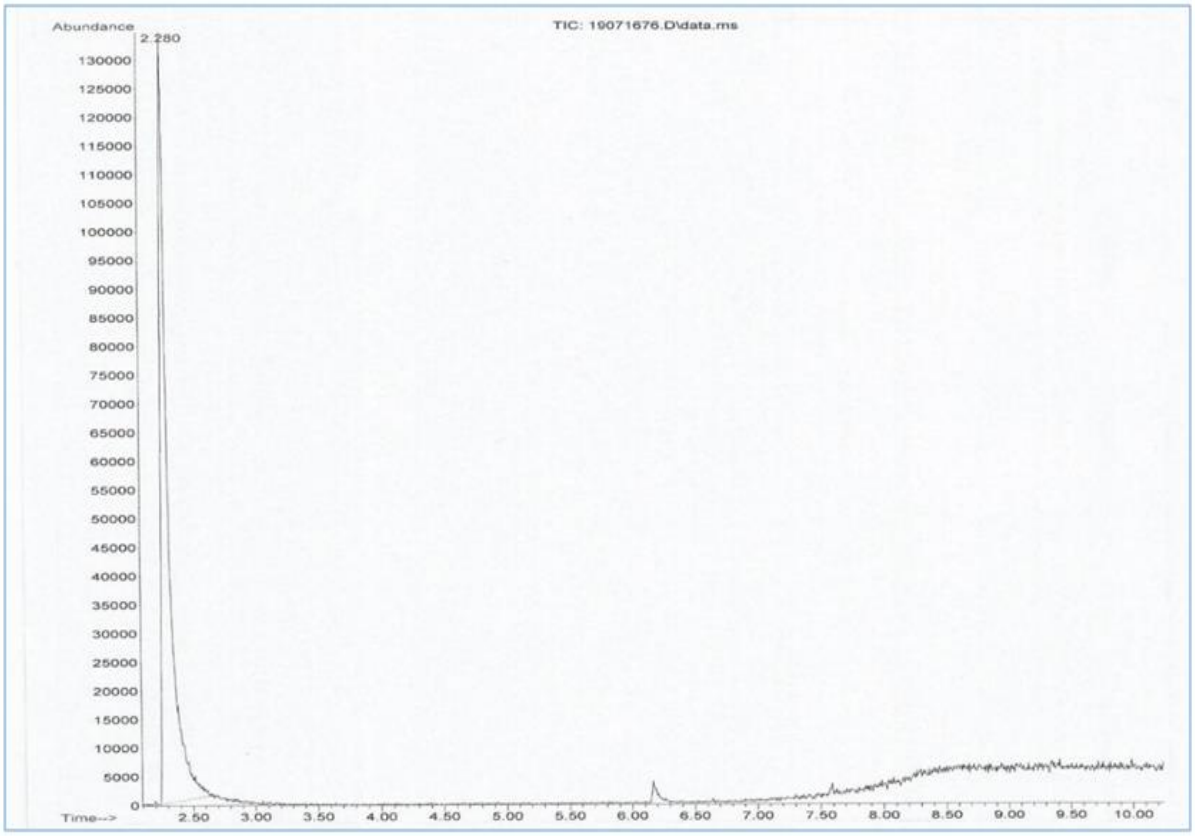

Fig. 4: Typical GC-MS chromatogram used to assess the volume of methanol required to recovery concentration of 0.050 mg mL^{-1} methadone with 0.040 mg mL^{-1} hexadecane as an internal standard showing the abundance (counts) against time (min) at elution stage 2 for 0.50 mL of methanol.

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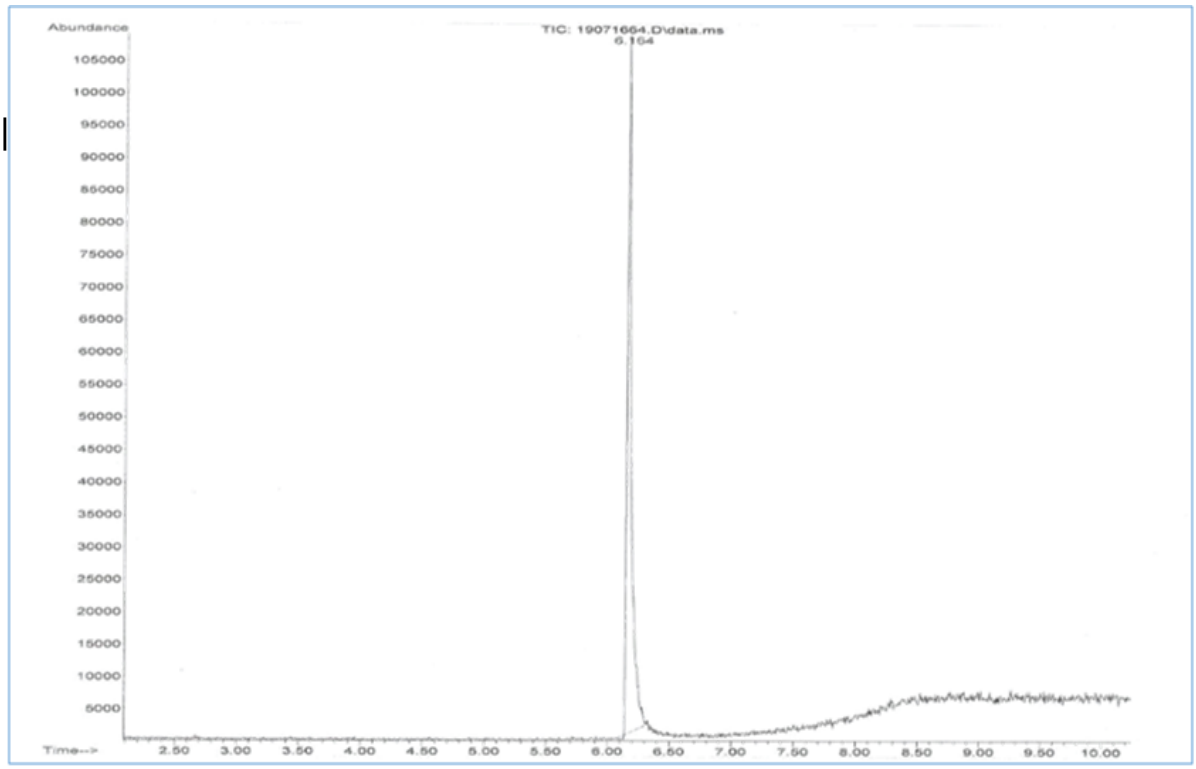

Fig. 5: Typical GC-MS chromatogram used to assess the volume of methanol required to recovery concentration of 0.050 mg mL^{-1} methadone with 0.040 mg mL^{-1} hexadecane as an internal standard showing the abundance (counts) against time (min) at elution stage 3 for 0.50 mL of methanol.

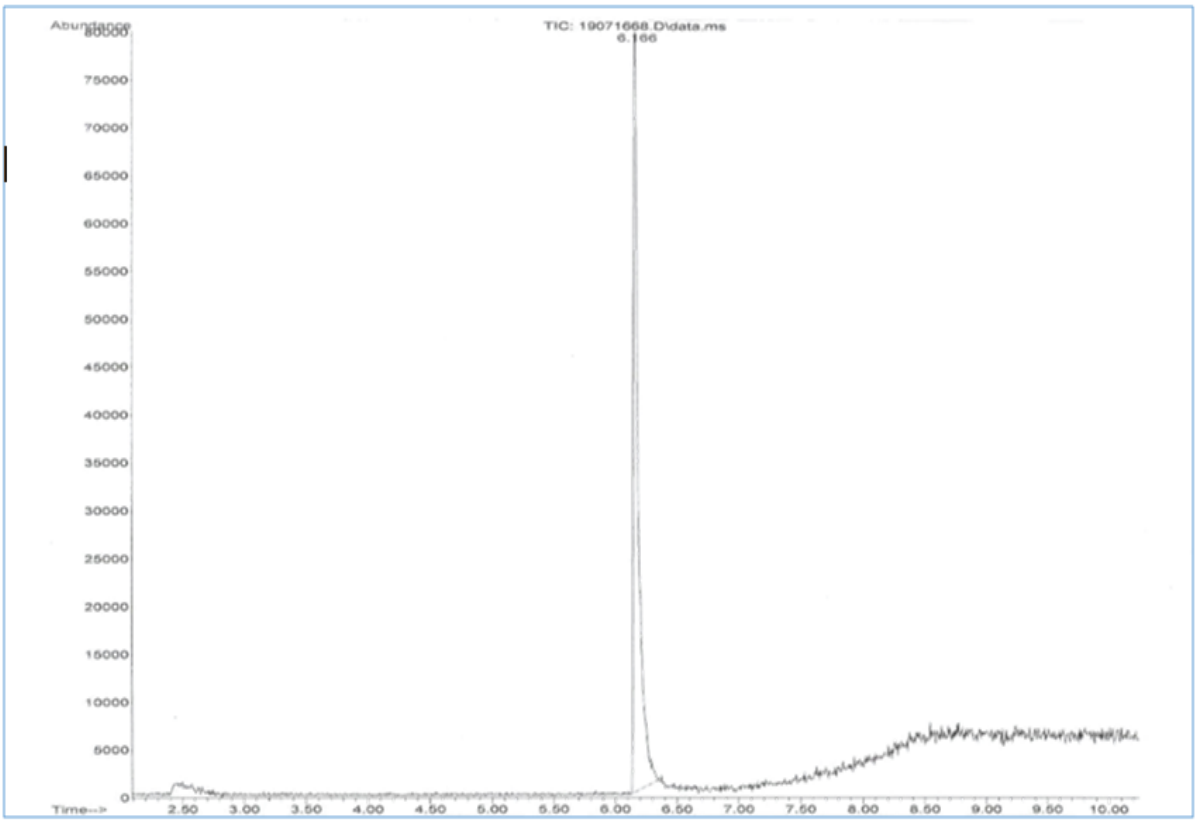

Fig. 6: Typical GC-MS chromatogram used to assess the volume of methanol required to recovery concentration of 0.050 mg mL^{-1} methadone with 0.040 mg mL^{-1} hexadecane as an internal standard showing the abundance (counts) against time (min) at elution stage 4 for 0.50 mL of methanol.

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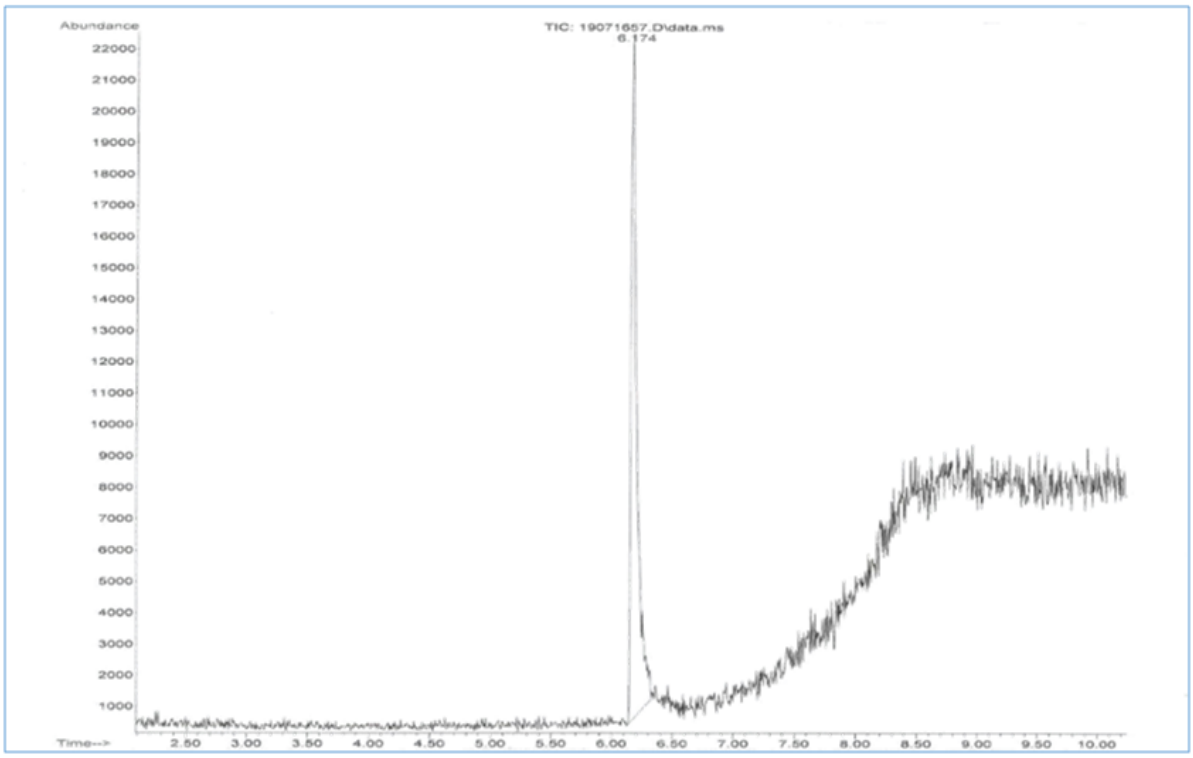

Fig. 7: Typical GC-MS chromatogram used to assess the volume of methanol required to recovery concentration of 0.050 mg mL^{-1} methadone with 0.040 mg mL^{-1} hexadecane as an internal standard showing the abundance (counts) against time (min) at elution stage 5 for 0.50 mL of methanol.

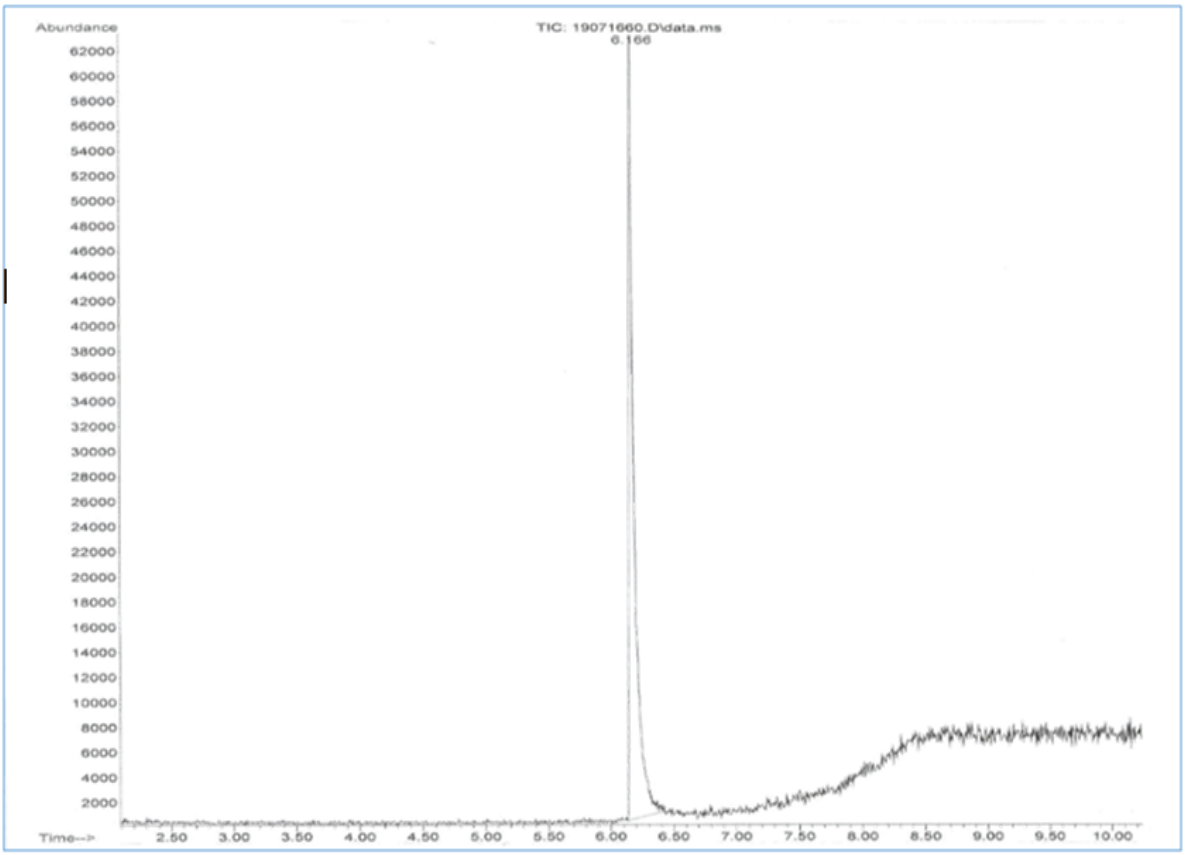

Fig. 8: Typical GC-MS chromatogram used to assess the volume of methanol required to recovery concentration of 0.050 mg mL^{-1} methadone with 0.040 mg mL^{-1} hexadecane as an internal standard showing the abundance (counts) against time (min) at elution stage 6 for 0.50 mL of methanol.

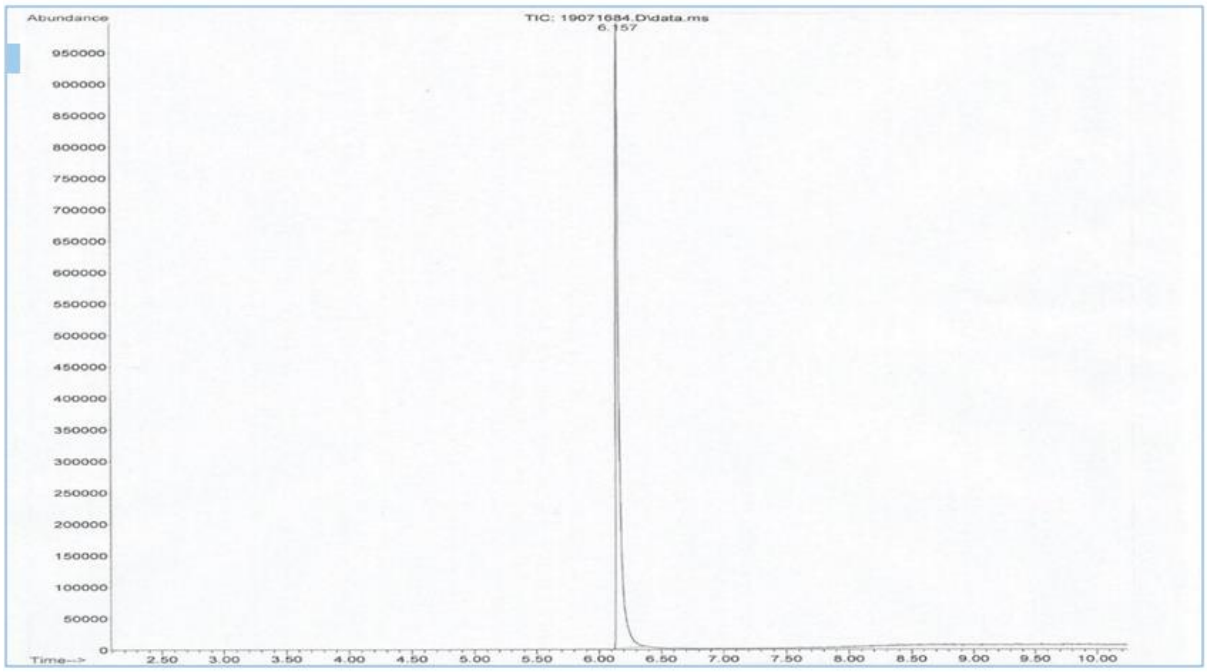

Fig. 10: Typical GC-MS chromatogram of urine spiked with concentration of 0.050 mg mL^{-1} methadone containing 0.040 mg mL^{-1} hexadecane internal standard eluting with 3 mL of methanol after evaporated to dryness under a stream of nitrogen at 40 ℃ followed by reconstitute with 0.50 mL methanol, showing the abundance (counts) against time (min).

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