Correlation of some Drugs of Abuse in Human Urine by EMIT and GC/MS Analysis

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Abstract:

Drug toxicology tests are most commonly performed on urine, since most drugs and their breakdown products are excreted in the urine at higher concentration. A total number of 449 inhabitant urine samples were collected from patients admitted to emergency hospital, Mansoura University (Nov 1999 to June 2005). These samples were analyzed by EMIT and GC / MS for benzodiazepine, barbiturate, opiate and cannabinoid. The aim of this work is to evaluate the usefulness of using urine immunoassay kits for detection of some drugs of abuse, and study the correlation between the results obtained by EMIT and GC / MS techniques. Results revealed that urine immunoassay kit is useful for rapid preliminary screening of abuse drug. GC / MS results confirm that 245 samples (54.56 %) are positive of the total number of samples. These positive samples by GC / MS were as follows; benzodiazepines; 159 (clonazepam, oxazepam, temazepam), barbiturates; 58 (thiobarbiturate, butabarbital, seconal) and opiates; 28 (methadone metabolite)). We conclude that GC / MS analysis must be done for accurate identification and confirmation of EMIT results. Also, GC / MS is recommended as the most suitable technique for obtaining optimum analytical results.

Keywords: Urine samples, EMIT, GC/MS.

1.0 Introduction:

Drugs may cause a direct physiological and psychological change in the body. Drugs of abuse are any drug or substance which if taken by any route (oral-snuffing-injection) will lead to mood change, psychological disturbance and can affect brain functions and level of perception (Gold frank *et al*, 1990). They alter the cultural environment and cause considerable concern as they are used by large numbers of young people during their reproductive period (Jones, 1990). Surveying the most common drugs and substances of abuse in Egypt it were found to be opiates, benzodiazepines, barbiturates, alcohol, cannabis and CNS-stimulants (Abdel-Magid and Salem, 1995). Most drugs of abuse are detectable by immunoassays, as far amphetamine, opiate, barbiturate, benzodiazepine, cocaine, P-C-P and cannabinoid. The concentrations of drugs are relatively high in urine, so it is the sample of choice for screening and identification of unknown drugs or poison. However the metabolites of these drugs must be identified in addition or even exclusively, (Maurer, 1992). Immunoassay techniques such as enzyme multiplied immunoassay technique (EMIT) are commonly used for drug screening in part because they are quick and require a small amount of samples. Gas Chromatography / Mass Spectrum (GC / MS) analysis was performed for identification, quantification and confirmation of the obtained results. The EMIT assay is a homogenous enzyme immunoassay technique used for the analysis of specific compounds in human urine. Once the urine sample has been identified as testing positive by a screening test, the specimen is retested with a more specific confirmatory test. The basic principle of confirming a positive drug test is to retest the same urine sample with different tests. The aim of this work was to evaluate the usefulness of using urine immunoassay kits for detection of some drugs of abuse, and study the correlation between the results obtained by EMIT and GC / MS techniques.

2.0 Materials and Methods:

2.1 Samples:

Four hundred and forty nine inhabitant urine samples were collected from patients admitted to poison unit at emergency hospital, Mansoura University (Nov 1999 to June 2005). Those patients were requested for drug screening; benzodiazepine, barbiturate, opiate and cannabinoid.

2.2 Reagents of EMIT:

Four EMIT drug assay urine (d.a.u.) kits for benzodiazepine, Barbiturate, opiate and cannabinoid were purchased from Syva company and prepared according to the manufacturer manual (EMIT, 1984).
- EMIT d.a.u. assay composed of:
Reagent A; antibody to a particular drug/ substrate for the enzyme (G6PDH).
Reagent B; enzyme - labeled drug.
- EMIT drug assay buffer concentrate.
- EMIT calibrator 0 (negative) and calibrator A levels 1(cutoff) and 2 (high), these calibrators are used in the calibration of the EMIT d.a.u. (benzodiazepine, barbiturate, opiate) assays where the cutoffs are nearly the same; however the cannabinoid kit has separate calibrator.
All reagents were refrigerated at 2 – 8 ºC for storage and allowed to equilibrate for at least two hours at room temperature before use.

2.3 Technique of EMIT:

Each sample was assayed by the four separate EMIT d.a.u. with the pipette diluter, 50 µl of the urine sample was added to 250 µl of buffer solution and mixed in a 2 ml disposable cup. 50 µl of reagent A and 250 µl of the buffer were added to the cup. After 30 sec. equilibration, 50 µl of reagent B and 250 µl of the buffer were added to the cup. The contents of the cup were immediately aspirated into the flow cell of the spectrophotometer. Absorbance readings were taken automatically at 15 and 95 sec. to calculate the absorbance difference (ΔA).

2.4 Identification method of drug abuse using GC / MS:

Extraction of urine samples (Ghanem, 2005)
Acid Extraction:
- One ml of urine was acidified with 100 µl of 1N HCl, followed by extraction with 5 ml chloroform.
- Shake well for 10 seconds and centrifuge for 5 min. at 3500 rpm.
- Aspirate and discard upper layer and filter organic phase through Whatmann filter paper No 4 in glass tube.
- Evaporate the extract to dryness, reconstitute with 100 µl chloroform.

Base Extraction:
- An aliquot of 700 µl of urine sample were alkalized with100 µl of NaOH 5 M followed by extraction with 150 µl of butyl acetate.

- Vortex the mixture for 10 seconds, then centrifuge for 5 min. at 11000 rpm.
-Evaporate butyl acetate extract to dryness, reconstitute with 100 µl   diethyl ether. Inject 1 µl of the extract injected into GC - MS (Hewlett Packard 6890 series) of ECD (Electron Captured Detector) as universal detector and examine in Wiley library.
- All chemicals used were of analytical grades.

Conditions of GC / MS (Maurer, 1992)
- Carrier gas (He)
-Capillary column; model No: HP19091Z-102, Hp-1 Methyl Siloxane, Length 25 m, diameter 200 µm, film thickness 0.33 µm.
- Flow rate 1.0 ml / min, Mode: split less,
- Thermal Aux 2 (MSD; Mass Spectrum Detector),
- Temp 280 ºC, Max temp 325 ºC.

Drug	Run Time min.	Average velocity of carrier Gas cm/sec	Oven Tem. ºC	Pressure of carrier gas (back inlet) psi
Benzodiazepine	14	41	110	22.2
Barbiturate	5	41	110	22.2
Cannabinoid	7	42	180	27.8
Opiate	4	42	200	29.3

Standard spectra auto tune, (Fig. 1) was done daily and before injecting the samples using internal standard Pentafluorotetrabutylacetate (PFTBA), three peaks appeared (69, 219 and 502 ± 0.1) with peak width (0.55 – 65).

Statistical analysis for evaluation of drug screen examination by EMIT and GC/MS was carried out by specificity and sensitivity for each drug using the following equations, (Mustafa, et al., 1989):
Specificity = (true negative / true negative + false positive) x 100
Sensitivity = (true positive / true positive + false negative) x 100

3.0 Results and Discussion:
The annual positive tests for each abused drug in (Mansoura area, Egypt) by EMIT system were; benzodiazepines (329), barbiturates (172), opiates (165), and cannabinoid (81). While, the positive tested samples by GC / MS Table (1) are as follows; benzodiazepines (159), barbiturates (58) and opiates (28). The spectra and distinctive ion scanning (m / z) of the positive drugs; benzodiazepines, barbiturates and opiates by GC / MS analysis are illustrated in Figs

(2a, 2b, 2c & 3a, 3b, 3c & 4). The administered drugs and their metabolites were identified in urine samples through analysis of their respective GC / MS spectra as follows; for benzodiazepines (clonazepam: m / z 280, 314; oxazepam: m / z 268, 239; temazepam: m / z 271), for barbiturates (thiobarbiturate: m / z 42, 144; butobarbital: m / z 156, 141, 55; Seconal: m / z 41, 168) and for opiates (methadone metabolite: m / z 276, 262, 105), Table (2). Evaluation of drug screen examination by EMIT and GC / MS was carried out by specificity for each drug as follows; benzodiazepines (63.04 %), barbiturates (77.4 %) and opiates (75.4 %) and also by sensitivity for each drug as follows; benzodiazepines (48.3 %), barbiturates (17.3 %) and opiates (8.97 %), Table (3).

Drug screening is preferably preformed in the urine (Frazer, 1992 and Walls *et al,* 1997), where the concentrations of drugs are relatively high and the time of detection is long, so that this choice is for screening and identification of unknown drugs or poison. However, the detection of drugs or their metabolic products in the urine indicate only the possibility, but not the certainty that those drugs were active at the time of accident. GC / MS is today the method of choice for systematic toxicological analysis in clinical and forensic toxicology. The screening can be performed using mass chromatography followed by a library search. Results of the present work revealed that urine immunoassay kit is useful for rapid preliminary screening of abuse drug. The qualitative examination by EMIT system of the collected samples indicates that positive tests are as follows; benzodiazepines (329), barbiturates (172), cannabinoid (81) and opiates (165), in the 499 samples for each test, Table (1). Moreover, EMIT use is generally limited for identification of drug abuse as a whole. In spite of the immunoassay rapidity, it must be confirmed by GC / MS analysis, especially for samples withdrawn from patients ingesting low dose of these drugs or those with high potency in small doses. The positive tested samples by GC / MS are as follows; benzodiazepines (159), barbiturates (58) and opiates (28), in the 499 samples for each test, Table (1).

Table 1: Comparison between Results of Positive Drug Tests by EMIT and GC / MS

Drug/ year	No of samples	Positive tests Benzodiazepines		Barbiturates		Cannabinoid		Opiates		Total No of Positive tests	
		EMIT	GC/ MS	EMIT	GC/M S	EMIT	GC/ MS	EMIT	GC/ MS	EMIT	GC/ MS
Nov1999	16	12	3	2	2	3	-	2	2	19	7
2000	176	115	59	69	20	25	-	43	5	252	84
2001	197	185	94	76	35	22	-	117	16	400	145
2002	16	-	-	15	-	9	-	2	-	26	-
2003	3	-	-	-	1	2	-	1	-	3	1
2004	18	3	2	2	-	16	-	-	1	21	3
April2005	23	14	1	8	-	4	-	-	4	26	5
Total	**449**	329	**159**	172	**58**	81	-	165	**28**	747	**245**

Fig. (1) Standard spectra of Auto Tune
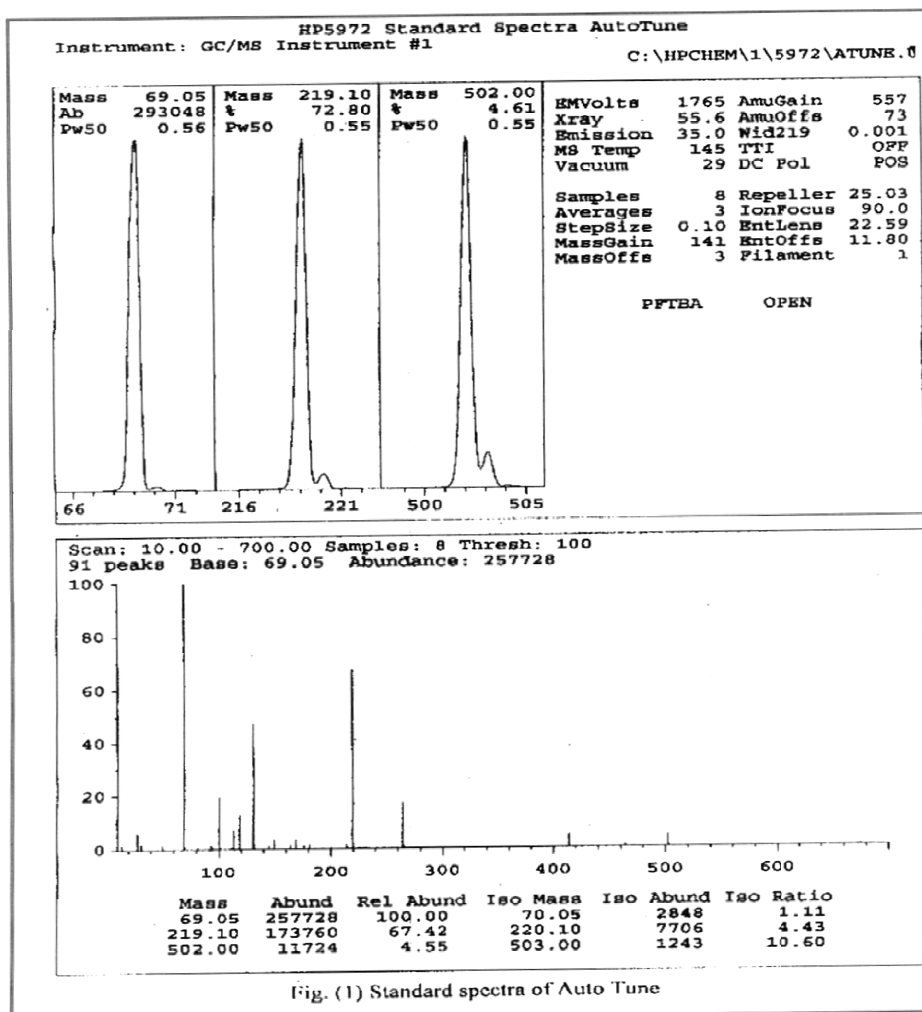
Table 2: GC/MS Ion Scanning

Group	Compunds	Selective Fragment Ions (amu)(m/z)*
Benzodiazepines	Clonazepam,	280, 314
	Oxazepam,	268, 239
	Temazepam	271
Barbiturates	Thiobarbiturate	42, 144
	Butobarbital	156, 141, 55
	Seconal	41, 168
Opiates	Methadone metabolite	276, 262, 105

m/z *: mass to charge ratio   amu: atomic mass unit

Oxazepam   C: \ DATABASE\WILEY275.L
Molecular formula: C15H11ClN2O2
Molecular weight: 286
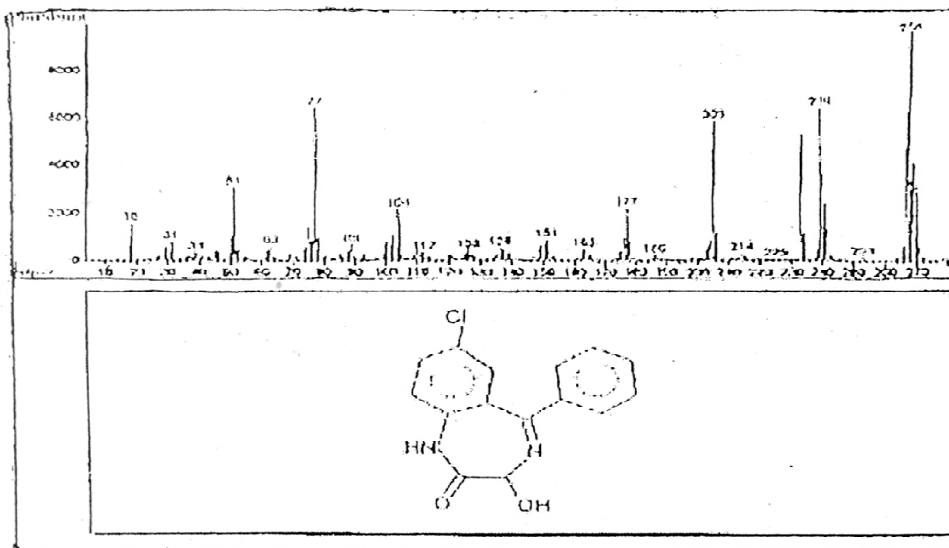
Fig. (2a) Spectra and structure of Oxazepam

Temazepam   C: \ DATABASE\WILEY275.L
Molecular formula: C16H13ClN2O2
Molecular weight: 300.067
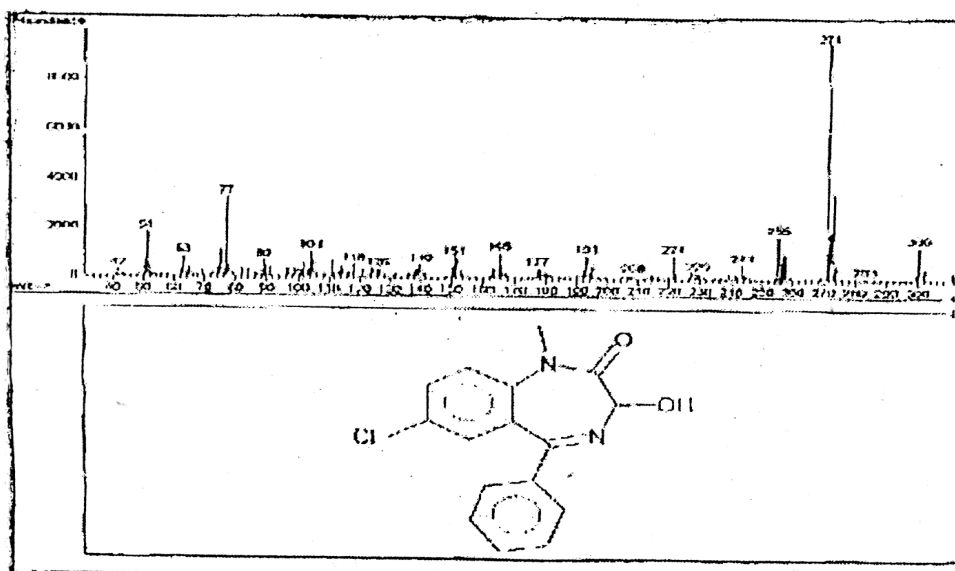
Fig. (2b) Spectra and structure of Temazepam

Clonazepam   C: \ DATABASE\WILEY275.L
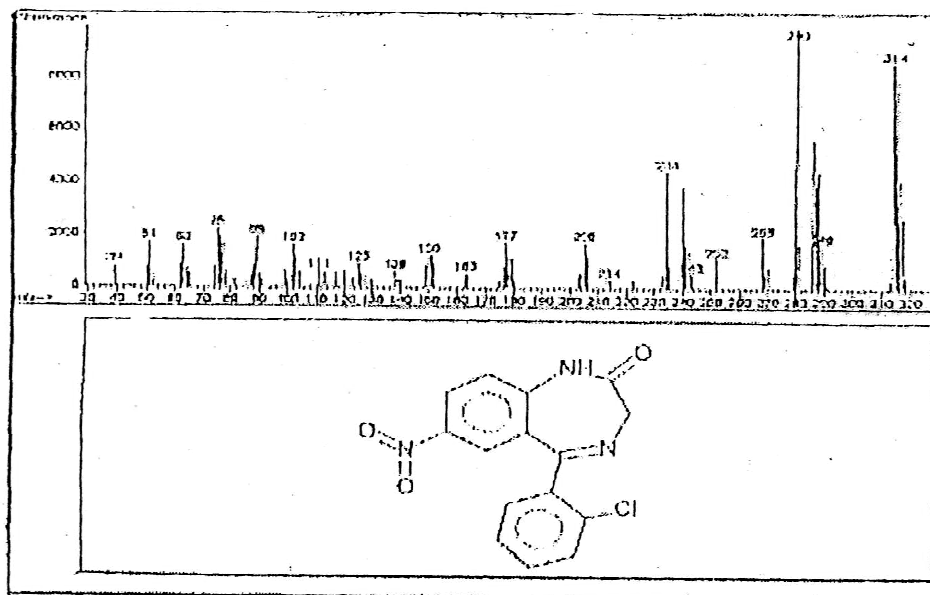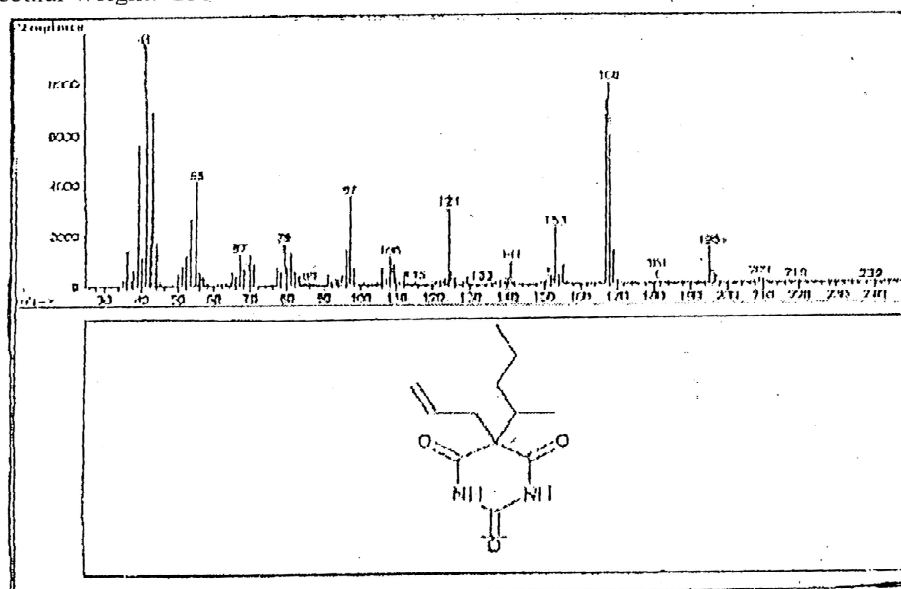Molecular formula: C15H10ClN3O3
Molecular weight: 315.041

Fig. (2c) Spectra and structure of Clonazepam

Seconal   C: \ DATABASE\WILEY275.L
Molecular formula: C12H18N2O3
Molecular weight: 238

(3a) Spectra and structure of Seconal
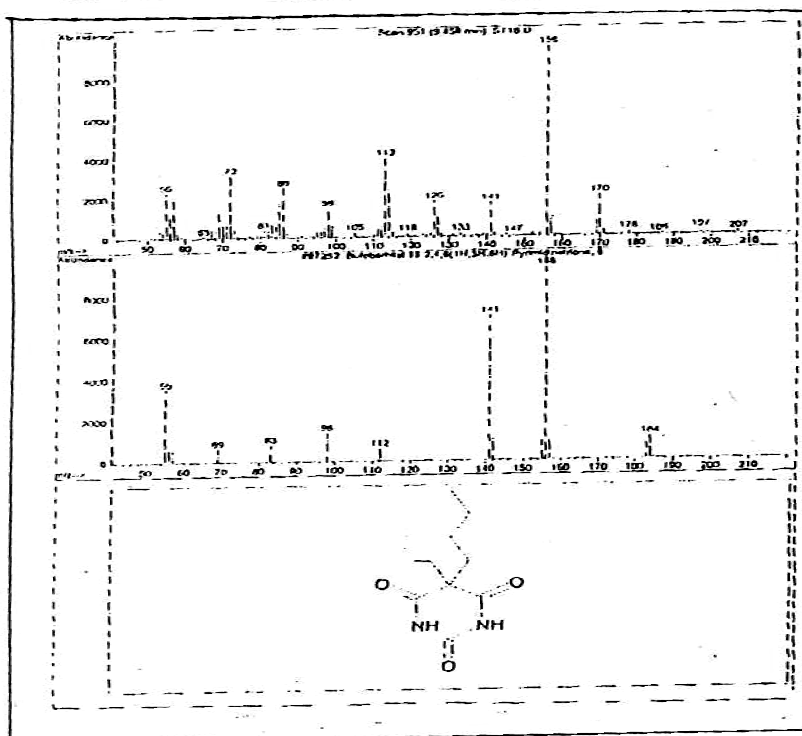
Butobarbital   C: \ DATABASE\WILEY275.L
:

Fig. (3b) Spectra and structure of butobarbital

Thiobarbitone   C: \ DATABASE\WILEY275.L
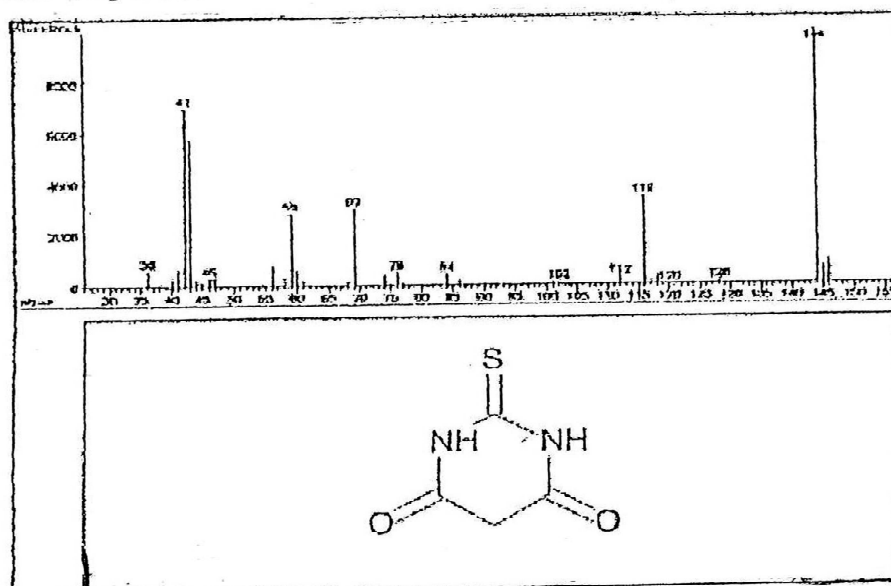Molecular formula: C4H4N2O2S
Molecular weight: 144

Fig. (3 C) Spectra and structure of Thiobaritone

Methadone metabolite C:\DATABASE\WILEY275.L
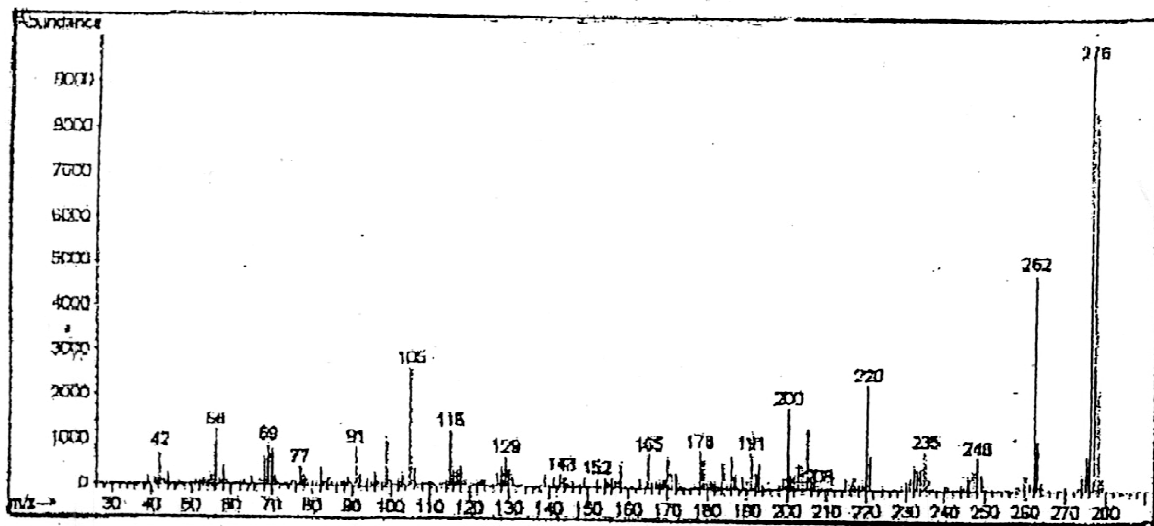Molecular formula: C20H23N
Molecular weight: 277.183

Fig. (4) Spectra of Methadone metabolite

It must be remembered that negative results of some drugs as cannabinoid using GC / MS analysis doesn't mean that those drugs weren't ingested, only they weren't detected due to their concentrations were below the sensitivity range of the assay used, the sampling time wasn't optimal or their clearance rate in urine is rapid. GC / MS distinctive ions of each category individually detected from the mass spectra of the corresponding drugs and their metabolites detected in urine samples as follows; for benzodiazepines (clonazepam: 280, 314; oxazepam: 268, 239; temazepam: 271), for barbiturates (thiobarbiturate: 42, 144; butobarbital: 156, 141, 55; seconal:41, 168) and for opiates (methadone metabolite: 276, 262, 105), Table (2). All chromatographic peaks have to

be identified because any of them may represent a potential poison. Positive signals and the identity of any of those drugs and/or their metabolites can be confirmed by visual or computerized comparison of the peaks underlying full mass spectra with reference spectra (Pfleger et al, 1992). Since GC / MS has been reported to be more specific than EMIT for benzodiazepines (63.04 %), barbiturates (77.4 %) and opiates (75.4 %) while, it is approximately equally in sensitivity (48.3 %) with EMIT for benzodiazepines and less sensitive than EMIT for barbiturates (17.3 %) and opiates (8.97 %), Table (3). So, we used it as a reference test for evaluation of drug screen examination. Moreover, on the basis of the previous results, GC / MS proved to be of higher specific in recognition of certain drugs, where 245 tests/samples were positive representing 54.56% of the total positive samples (449) and 32.79 % of the total positive tests (747) by EMIT. Thus GC / MS analysis help to solve the false positive results obtained by EMIT.

Table 3: Specificity and sensitivity for each drug detected by GCMS analysis

Drugs	Sensity (%)	Specifity (%)
Benzodiazepines	48.3	63.04
Barbiturates	17.3	77.4
Opiates	8.97	75.4

4.0 Conclusion:

In conclusion, this work revealed that EMIT is a rapid preliminary detection test for drug screening in the urine. The data obtained revealed the failure of EMIT to identify the specific drug or its metabolite as do GC / MS. Hence, in spite of the rapidity of EMIT, GC / MS analysis must be done for accurate identification

and confirmation of EMIT results. It is recommended to extend the use of EMIT for drugs screening in urine when a rapid diagnosis is needed. Also, GC / MS is recommended as the most suitable technique for obtaining optimum analytical results.

5.0 References:

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