Development of Systematic Toxicological Analysis Using GC-MS in the Identification of Drugs in Blood for Clinical and Forensic Purposes

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ABSTRACT

A rapid auto-identification method was developed to reduce the time required for unknown detection in defined groups of compounds in postmortem biological materials. This was achieved by the creation of an excel sheet containing retention time and retention index of *n*-alkane mixture ($C_{15} - C_{36}$). Then, a calculated retention index and the retention time for a list of compounds with their target ion, two reference ions and their ion ratios were saved in a new method table. The new system will automatically search for and identify any compound in the post-run Gas Chromatography – Mass spectrometry data. The developed method showed a high sensitivity and specificity.

Keywords: Toxicological analysis, GC-MS Clinical and Forensic purposes.

INTRODUCTION

The ability to perform a comprehensive and systematic analysis of specimens, for the presence of chemicals of toxicological importance, is termed systematic toxicological analysis (STA). Also, STA is defined as: the undirected chemical-analytical search for potentially toxic substances whose presences are uncertain and whose identities are unknown and is obviously required if little or no information is available as to which toxic agent is involved, the so-called General Unknown Case (GUC) [1, 2]. The choice of the method in analytical toxicology depends on the problem which has to be solved. The analytical strategy often includes a screening test and a confirmatory analysis. Positive results must be confirmed by a second independent method that provides the highest level of confidence in the result [3]. The availability of automated rapid and reliable methods for the STA of drugs and poisons in biosamples is of great importance in clinical and forensic toxicology laboratories [4].

Unfortunately, not all substances can be detected with one drug screening method. The presence of different physiochemical properties affects the ability to extract substances from biological matrices; while thermal stability, polarity and detector sensitivity affect the detectability of drugs in a variable chromatographic system [1]. The introduction of chromatographic techniques in analytical toxicology in the late 1950s and the development of rapid-scanning (1 scan/s or higher) multichannel spectroscopic detectors that is able to record the whole spectrum of the column elute represented the keys in the evolution of systematic toxicological analysis. A great effort was made towards the standardization of chromatographic data in order to make this information usable on an inter-laboratory basis [4-6]. The separation power of capillary gas chromatography and the selectivity of mass spectrometry make GC-MS the technique of choice for STA [4]. The simplicity of the information

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contained in a mass spectrum; in addition to the availability of large databases of mass spectra of reference compounds [7-8], both allow a fast and reliable computer-aided identification of unknown compounds based on the method of fingerprint, the so-called Library Search. Moreover, by including single-step broad spectrum derivatization and a reliable method for automated detection and identification of unknowns in GC-MS data files, most of the weak points of the technique can be overcome [4].

Today, it is well recognized that GC-MS represents the "golden standard" for drug analysis because of its selectivity and identification power [4, 9]. On the other hand, GC has well known weak points as a separation technique compared to HPLC [10]. To develop a fully automated method for systematic toxicological analysis based on GC-MS, development must go beyond sophisticated sample preparation to focus also on the extraction of relevant analytical information (mass spectra of analytes) from the chemical noise [4]. To perform proper systematic toxicological analysis, all peaks in a run by GC-MS analysis have to be searched against spectral libraries [8], and because large numbers of spectra are obtained by every GC-MS run, this is a very time consuming task. Therefore, a method for retrieving target compounds via reconstructed mass chromatograms was proposed by Maurer and is now widely employed in forensic laboratories [9]. For any screening system there are limitations with respect to the ability to detect drugs and poisons. Awareness of the strengths and limitations is of critical importance in any systematic analysis of specimens for the presence of drugs[1].

Within the last ten years attempts in GC-MS-STA procedures were concentrated more on automatic detection of drugs and poisons, either by automatic identification from the standard libraries or recognition of poisons by automated subtraction procedure [4, 11], using computer-assisted identification. Although these procedures gave STA more steps forward, they still had some limitation and prerequisites [4]. In this work, we tried to establish an STA method that helps in the identification of unknown in common groups of drugs and poisons regardless of the method of pretreatment and inter-laboratory variations.

INSTRUMENTATION

Analysis was performed on Shimadzu 2010 Gas chromatography with a Shimadzu Auto-injector AOC 20 connected to a QP2010 MSD (Quadrupole Mass Spectroscopy Detector) (Shimadzu, Kyoto, Japan). In the gas chromatograph, an HP-35 (Hawellet Packard) Column (30 m x 0.32 mm, 0.25 µm film thickness) was installed with a constant flow of high-purity helium (99.999%, UAE) at 1 ml/min. The temperature programming used was as the following, the initial column temperature was set to 150 ºC for 1 min, then increased to 280 ºC by 15 ºC /min, with a total running time of 25 minutes. Split injections were performed with ratio: 10.5, the injection-port temperature was 280 ºC, interface temperature was 280 ºC and that of the ion source was 200 ºC and Solvent cut time was 2.4min. Full-scan EI (Electron Impact) spectra were recorded from 30-550 m/z (Mass/charge) with 2 scans per second. Shimadzu GCMSolution® version 2.4 was used in data acquisition and integration.

MATERIALS, METHODS AND TECHNIQUES

All chemicals used were analytical or HPLC grade. Drug standards were available in the toxicology laboratory/ Faculty of Medicine; the University of Jordan; where the method was developed. Postmortem blood and vitreous humor samples were extracted using liquid-liquid extraction method, which included taking one ml of the sample into a 15 ml glass tube, then one ml of 0.5 M NaHPO4 and 5 ml of ethylacetate as extraction solvent that contains the IS (Lamotrigine 200ng/ml) were added and mixed for 5 minutes. The mixture was centrifuged for 2 minutes at 2000/rpm. The organic layer was taken into another clean 15 ml glass tube and evaporated at 40ºc using Dri-Block heater under gentle nitrogen gas flow. After dryness, it was reconstituted with 100 µl acetonitrile and transferred into glass insert of auto-injector vial. When the extracted sample was not analyzed directly, it was tightly closed and stored at -20 ºC. The drug standards studied were prepared at 1µg/ml methanol concentration.

Retention index

The standard solution to calibrate the retention index

calculation contained a mixture of the *n*-alkanes C_{15} - C_{36} in methanol. A 100 ng per compound were injected to GC-MS according to the above method. The integrated data, with the retention time (RT) and retention index (RI) for each alkane was saved. New Microsoft Office excel sheet (Microsoft Office Excel 2003®) was created with RT and RI of each alkane.

Development of drug identification

Each drug was injected to GC-MS according to the same method. Once acquisition was finished, the post-run analysis program data file (part of GC-MS Software) was opened. In the qualitative menu, the data file of *n*-alkane was uploaded to auto-calculate the retention index for each scan. The compound was identified by using Wiley and NIST (National Institute of Standard and Technology) libraries. The RI of the compound that appeared in the chromatogram was transferred into the excel sheet created before with its name and RT. The peak of the compound was manually integrated, and the integration parameters and RT were exported to the qualitative table (TIC section "Total Ion Chromatogram"). The qualitative table contains two parts; spectrum process table and TIC. The peak number that referred to the drug standard was registered to spectrum process table by right clicking on it, and its name then typed.

Parameters determination

After finishing the above step, the compound table within the wizard method was opened and the target ion and two reference ions then determined. The name of the compound will be automatically loaded from spectrum process table. The ions were selected easily by double clicking on them in the spectrum. The compound table was then saved as method file. After the creation of the compound table, more standards were feed to it according to the screening method required or designed, by editing the compound table. The name of the new standard was entered, and its spectrum determined and registered. "Set the compound information" was selected, (an option in the menu) and the same compound table wizard appeared. Again, the target ion and two reference ions and RT were identified. At the end, the compound table with all standards names and their RT, target ions, reference ions and reference ion ratios was saved. The RI and the names of standard drugs or compounds were recorded on the same excel sheet. In the excel sheet, both the RT and RI of each compound were connected with the RT and RI of *n*-alkane. In that way, the software will automatically correct the RT of the compounds if it changed due to any variation in GC parameters. What was needed is to do that manually by running the *n*-alkane mixture, then the RT for each alkane, and the newly corrected RT for the drugs was copied and pasted in the RT column of the compound table of the new method.

Table (1): The table show the result of 364 samples, the number of true negative and true positive results for each compound, the number of false positive (FP) and false negative (FN) results that auto-identified by the developed method, the percentage of FP and FN result, the sensitivity and specificity for each compound, the positive and negative predictive value (PPV), and (NPV); respectively.

Compound Name	# of negative sample	# of positive samples	# FP	# FN	% of FP	% of FN	sensitivity %	Specificity %	PPV %	NPV %
Nicotine	292	72	2	0	2.8	0.0	100.0	99.32	97.3	100
Ephedrine	362	2	0	0	0.0	0.0	100.0	100	100	100
Pentobarbital	349	15	1	0	6.7	0.0	100.0	99.7	93.75	100
Acetaminophen	197	167	4	0	2.4	0.0	100.0	98.01	97.7	100
Cotinine	203	161	2	0	1.2	0.0	100.0	99.02	98.8	100

Compound Name	# of negative sample	# of positive samples	# FP	# FN	% of FP	% of FN	sensitivity %	Specificity %	PPV %	NPV %
fluoxetine	363	1	0	0	0.0	0.0	100.0	100	100	100
Pentothiobarbital	355	9	0	0	0.0	0.0	100.0	100	100	100
Lidocaine	331	33	1	2	3.0	6.1	94.3	99.7	97.1	99.4
Tramadol	361	3	0	0	0.0	0.0	100.0	100	100	100
Caffeine	34	330	4	0	1.2	0.0	100.0	89.47	98.8	100
Theophylline	346	18	1	0	5.6	0.0	100.0	99.7	94.7	100
Procyclidine	361	3	1	0	33.3	0.0	100.0	99.7	75	100
Amitriptyline	363	1	0	0	0.0	0.0	100.0	100	100	100
benzhexol	364	0	0	0	0.0	0.0				
biperiden	364	0	0	0	0.0	0.0				
Imipramine	363	1	0	0	0.0	0.0	100.0	100	100	100
Carbamazepine	357	7	0	0	0.0	0.0	100.0	100	100	100
Phenytoin	341	23	2	1	8.7	4.3	95.8	99.42	92	99.7
Lorazepam	364	0	0	0	0.0	0.0				
Diazepam	362	2	0	0	0.0	0.0	100.0	100	100	100
Lamotrigine	364	0	0	0	0.0	0.0				
Midazolam	362	2	0	0	0.0	0.0	100.0	100	100	100
Bromazepam	364	0	0	0	0.0	0.0				
Quinine	364	0	0	0	0.0	0.0				
Papaverine	364	5	0	0	0.0	0.0	100.0	100	100	100
Clonazepam	364	0	0	0		0.0				
Diltiazem	364	0	0	0	0.0	0.0				
Alprazolam	364	0	0	0	0.0	0.0				
						Average	99.5	99.16	97.11	99.95

Systematic drug and poison identification

After the development of the above method parameters, the extracted unknown sample was injected to GC and integrated into GCMSolution® post-run software, the saved method file (compound table) was loaded. The software searched for the drug in the compound table and automatically checked for the target ion, reference ions, reference ions ratio, RT and RI. If the drug is present, then the software will detect it automatically and it will appear with different color in the compound list as in the figure 1.

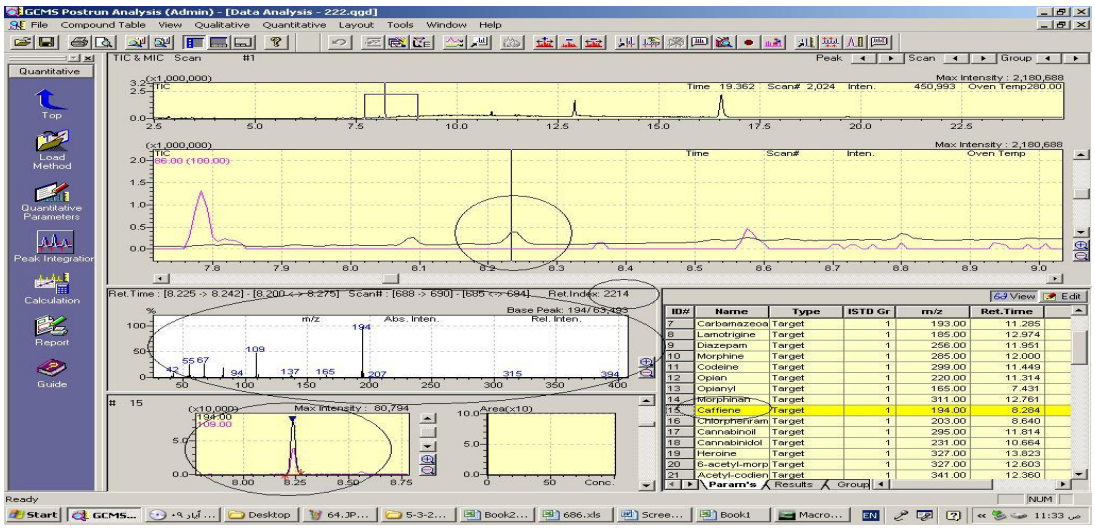

Figure (1): Automatic identification of the substance by the newly developed method.

RESULTS AND DISCUSSION

364 postmortem blood and vitreous humor samples were extracted and analyzed by the developed method. Firstly, the compounds were identified by the auto-system as above. Then a manual check for each drug (peak) was done. The result showed a high sensitivity for most detected compounds (100%) except lidocaine 94.3% and phenytoin 95.3%, with average sensitivity of 99.5% as shown in table 1. Also, the method had an excellent specificity of 99.16%. The positive predictive value (PPV: positive result for the truly positive sample) was 92-100%, except for procyclidine where it was 75% (n=3). The average of PPV was 97.1%. The negative predictive value (NPV: negative result for the truly negative sample) ranged from 99.3 to 100% with an average of 99.95%.

This method was developed mainly for weak bases and neutral compounds that are GC-MS compatible and required no derivatization, however the same procedure is applicable for all compounds in systematic toxicological analysis with no modification. For polar and GC incompatible compounds, the RT and calculated RI will be for the derivatives. So, after the first step in extraction, the sample may split into two portions: one is to be derivatized and the other is to be injected directly to GC. More improvement in the detection and identification power of this method is expected if solid phase extraction is used [9] and a cut-off limit for each compound is created. The reproducibility of the extraction method is not a prerequisite as with other studies [11] as long as the unknown is appearing in sufficient concentration in the extract.

This method was developed by using simple software but with high identification power. Most previously developed methods were using either newly developed software for a specific purpose [11], or a very sophisticated method of sample preparation including SPE (Solid Phase Extraction); or target compound identification or information subtraction software [4, 12]. The identification power is enhanced in this method by using a reference ion and two target ions, since TIC is less sensitive to low concentration. Additionally, the method is highly applicable in most toxicology laboratories having GC-MS. The main limitation of this method is that, pre-defined groups of drugs and poisons are required. This means that, it is not a general method for totally unknown compounds, but it enhances a rapid and simple identification of the poisons.

CONCLUSION

In conclusion, the presented method will help to shorten the time consumed to identify an unknown poisons in specific groups of compounds in systematic toxicological analysis, and could be extended to include more groups that share the same physiochemical properties or commonly encountered groups in specific clinical or postmortem intoxication circumstances.

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تطوير منهجية لتحليل السموم للأستعراف على الأدوية في عينات الدم لتطبيقات سريريه وجنائية باستخدام غاز الكروماتوغرافيا المرتبط بمقياس الكتلة

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ملخـص

تم تطوير طريقة سريعة للاستعراف الذاتي لتقليل الوقت اللازم للكشف على مواد غير معروفه في العينات البيولوجية، من ضمن مجموعة معرفة من المركبات الكيميائية. وقد تم الحصول على ذلك من خلال تطوير طريقة نموذج اكسل مصمم يحتوي على أزمنة الاحتباس و دليل الاحتباس لمخلوط الالكينات (C15-C36) ويليها دليل الاحتباس المحسوب وأزمنة الاحتباس لقائمة من المركبات الكيميائية وايوناتها المستهدفة، بالإضافة إلى ايونات مرجعية ونسبهم.

وتعمل الطريقة المطورة بالبحث والكشف اتوماتيكياً عن أي مركب كيميائي في المعلومات التي يتحصل عليها من جهاز الغاز الكروماتوغرافيا المرتبط بمقياس الكتلة (GC-MS). وتميزت الطريقة المطورة بنوعية وحساسية عالية.

الكلمـات الدالـة: تحليل السموم، تطبيقات سريرية وجنائية، غاز الكروماتوغرافيا، مقياس الكتلة.

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