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Carboxamide Synthetic Cannabinoids: As a Significant Causes of Related Death in Kuwait (Case Studies)

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Abstract

One of the most common New Psychoactive Substances (NPS) abuse in worldwide is the Synthetic Cannabinoids (SCs) which is seems to be a global problem. These compounds were originally developed to study the structure and function of cannabinoid receptors, but in recent years, they have emerged as drugs of abuse. Synthetic cannabinoids, most commonly known as Spice or K2, which is a mixture of herbs and spices that are sprayed with a chemical with similarities to THC, the mind altering ingredient found in marijuana.

Chemical analysis shows that the active ingredients in these drugs are synthetic chemicals with dangerous toxic effects. Beyond that, because the chemical composition of these products are unknown, users have no idea what chemicals they are putting into their bodies or what the effects will be, where herbs can be sprayed unevenly, and the potency can vary wildly.

Synthetic cannabinoids can be toxic. As a result, drug abusers who smoke these products their bodies can be affected seriously, different symptoms can be occurred and varied according to amount and kind of drugs consumed, such as: Rapid heart rate, vomiting, agitation, confusion, and hallucinations. Some have to get help from emergency medical services or in hospital emergency departments or intensive care units.

The aim of this study is to look after different kinds of synthetic cannabinoids in Kuwait, and to analyze and identify these compounds which is spread so quickly between drugs abusers, by using high performance Liquid Chromatography (LC/MS/MS) technique for both blood and urine samples.

In the year 2015, the drug control department in Kuwait notice that there is new drug addiction between the young adults and started seeing people coming into emergency rooms saying they smoked marijuana, in fact it is not marijuana it is the new drugs called synthetic cannabinoids and the common street name in the Arabian Gulf area is "Chemical", due to the chemical sprayed to herb that is give more powerful effect than natural THC.

During this time the new drugs has no regulation until September 2016 where all addiction and trading of these compounds and its derivatives become illegal.

Since that time, forensic toxicology laboratory in ministry of interior in Kuwait is keep looking after all techniques and analysis that is used to detect and identify these synthetic cannabinoids, especially in biological samples with a screening and confirmation test.

The majority of synthetic cannabinoids founds in biological samples (urine, and blood) were members of indazole-3-carboxamide family. Members from indazole-3-carboxamide family identified in Kuwait were 5F-ADB, FUB-AMB, AB-FUBINACA, AB-CHMINACA, MDMB-FUBINACA, 5F-AB-PINACA, AB-PINACA, and 5F-AKB48 which is also known as 5F-APINACA.

The most common synthetic cannabinoids were 5F-ADB, FUB-AMB, AB-FUBINACA and AB-CHIMINACA; various mixes of two, three, types of synthetic cannabinoids were identified.

The addictions of these compounds are responsible for hundreds of deaths every year. It affects not only individual users, but also their families and communities. Thus, it's important in gulf region to have this kind of studies related to new designer drugs.

In this work, three postmortem cases were analyzed and identified first by screening immunoassay test followed by confirmation by LC/MS/MS techniques, where a synthetic cannabinoids were confirmed. Combination of two or three kinds of these drugs with other narcotics such as: (opium, benzodiazepines, methamphetamine, and others) could be fatal.

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Introduction

Synthetic Cannabinoids (SCs) are a group of substances in the world of designer drugs that have become increasingly popular over the past few years. Synthetic cannabinoids are a chemically diverse group of compounds functionally similar to THC. Since first appearing on the world market a few years ago these compounds have evolved rapidly. Most of these substances appear to be manufactured in China. After being shipped in powder form to worldwide, the chemicals are typically added to plant material, packaged for sale as "legal high" products and often misleadingly labeled "not for human consumption".

SCs are usually smoked. Recently several countries have also reported finding the substances in products that look like cannabis resin either in branded "legal high" products or simply misrepresented as cannabis resin on the illicit market. This development is likely to be a response to the popularity of cannabis resin in many countries [1-3].

The number of synthetic cannabinoids, their chemical diversity and the speed of their emergence make this group of compounds particularly challenging in terms of detection, monitoring, and responding. Suppliers simply aim to mimic the effects of THC. In essence, this makes each synthetic cannabinoid disposable. When one synthetic cannabinoid is, or is about to be legally controlled manufacturers can have one or more replacement substance ready for sale [4].

Little is known about how these substances work and their toxic effects in humans. However, their use has caused many serious poisonings and even deaths, sometimes these have manifested as outbreaks of mass poisonings. It is possible that, along with being highly potent, some may also have long half-lives, potentially leading to a prolonged psychoactive effect. In addition, it appears that at least some of these substances have an effect on other physiological functions in the body beyond effects on the cannabinoid receptors [5-7].

Synthetic cannabinoids undergo extensive metabolic conversion. Consequently, both blood and urine specimens may play an important role in the forensic analysis of synthetic cannabinoids. The importance for accurate analysis of SCs in human biological matrices is evident and continues to be especially challenging due to their chemical structures being constantly modified. Many methods have been published recently for the analysis of SCs in human biological samples [8-10].

This study provides an overview of the analytical methods used for the analysis of SCs and their metabolites in biological specimens with a special focus on chromatographic analysis and sample preparation. Liquid chromatography assay is the most commonly used for confirmation purposes of SCs and their metabolites in biological matrices. In blood analysis of SCs must be very sensitive. In urine, SCs have extensive metabolism pathways; therefore the main target compounds are their hydroxyl and carboxyl metabolites.

Materials and Methods

Chemicals and standards

(a) Standards: All synthetic cannabinoids standards were purchased from Lipomed (Arlesheim, Switzerland) and Chiron AS (Trondheim, Norway). Most of them received as powder of 10 mg sealed vials, and few received as liquid of 1 mg/1 mL of calibrated solution in methanol (ampoules).

(b) Chemicals: β-Glucuronidase (Escherichia coli type) was purchased from Roche biomedical (Mannheim, Germany), methanol; water and acetonitrile were LC/MS grade from Fisher Chemical, while phosphate buffer and ethyl acetate were purchased from Sigma Aldrich. Other chemicals are available in toxicology laboratory.

Immunoassay screening

(a) Microline Urine Test (K3/AB-PINACA): The strip is made for the detection of synthetic cannabinoids in human urine, the tests were purchased from NaroCheck (France), the results occurred in 5 min with 99% reliability, and cut-off =10 ng/mL. The time of detection is about 2 to 3 days after consumption.

(b) Randox Screening (urine and blood): Randox immunoassay testing offers rapid separation of presumptive positive and negative specimens, prior to more costly and time consuming chromatographic confirmation. Evidence investigator biochip array technology is used to perform simultaneous detection of multiple analytes from a single sample. The core of the technology is the randox biochip; a solid state device with array of discrete testing regions containing immobilized antibodies specific to different drugs of abuse compound classes. The Randox DoA V Urine kit (Randox laboratories Limited, 55 Diamond Road, Crumlin, County Anntrim, UK) used in this paper employs a competitive chemiluminescent immunoassay, where the drug in the specimen and drug labeled with Horse Radish Peroxidase (HRP) are in direct competition for the antibody binding sites. Increased levels of drug in a specimen will lead to reduced binding of drug labeled with HRP and thus a reduction in the chemiluminescent signal emitted. The light signal generated from each of the test regions on the biochip is detected using digital imaging technology and compared to that from a stored calibration curve. The cutoff concentration of a drug or its metabolites in the matrix established for assigning negative and positive specimens. In an immunoassay, cutoff concentrations can be selected at the assay's optimal sensitivity, selectivity, and efficiency reducing the number of false positive and false negative specimens [11,12].

Extraction and samples pretreatment

(a) Sample pretreatment: Urine samples required pretreatment prior to Solid Phase Extraction (SPE) cleanup because synthetic cannabinoids are excreted in the urine as glucuronide conjugates. In the pretreatment procedure, the glucuronide conjugates were hydrolyzed to enhance extraction and detection by LC/MS/MS. The pretreatment procedure was performed as the following methodology: 2 mL of 100 mM acetate buffer (pH 5.0) and 50 mL of β-glucuronidase were added to each 1 mL sample of urine. The samples were then vortexes for 30 s, heated to 65°C for 1 h to 2 h, and allowed to cool [13-15].

The whole-blood samples or Plasma 500 µL were placed in glass vials, and then the samples were precipitated with acetonitrile. For this purpose, 500 µL of iced acetonitrile were added dropwise. During the addition, the sample was continuously mixed on a vortex mixer. In the next step, the samples were mixed for 5 min and centrifuged

at 13,000 rpm for 5 min. The organic solvent was transferred to 2-mL glass vials and then the acetonitrile was evaporated to dryness under an air stream at 30°C. The dry residues were dissolved in 100 µL of a mixture of 0.1% formic acid in acetonitrile/0.1% formic acid in water (1:4, v/v), and the solution was transferred to inserts for autosampler vials. The injection volume was 10 µL [16].

(b) Solid Phase Extraction (SPE) protocol: The CHROMABOND Column C18 volume was 3 mL and 100 mg sorbent weight (Macherey-Nagel, Düren, Germany) was used. Columns were set on a glass block vacuum manifold and the pretreated samples were loaded and the vacuum (10 mmHg) was applied for a slow drop-wise sample flow. Sample test tubes were washed with 3 mL 100 mM acetate buffer (pH 5.0), followed by a wash with 3 mL of 25% methanol in 100 mM acetate buffer (pH=5.0). Columns were then dried under full vacuum (10 mmHg) for 10 min. The collection rack was inserted with the test tubes into the manifold and eluted to retain compounds with 1 mL × 3 mL of ethyl acetate. Afterward, the SPE eluent was evaporated to dryness at 45°C under a gentle stream of nitrogen, and reconstituted in 100 mL of 50% methanol in deionized water. The extract was vortexes for 30s and transferred to inserts held in 2 mL autosampler vials for LC/MS/MS analysis [17-19].

LC/MS/MS setting and protocol

A Q-Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany) was used to confirm the results generated from the screening test of the biological urine and blood samples. The Q Exactive system provides very good analytical performance in terms of reproducibility, linearity, and signal-to-noise, and addresses an extremely wide range of masses.

The mass spectrometer is a Benchtop LC/MS/MS system that combines quadruple precursor ion selection with High-Resolution, Accurate-Mass (HRAM) Orbitrap detection. Samples (5 µL) were injected in a 2.6 mm Accucore™ Phenyl-Hexyl column (100 mm × 2.1 mm) and the LC column was heated to 40°C. Analytes were resolved at 0.5 mL/min using a mobile phase consisting of two solvents. The Mobile phases for LC-Screening are (Phase A: H_2O, $[NH_4]$+[HCOO]- 2 mM, 0.1% HCOOH), for 1 L mobile phase A use 1 L of water and add 126 mg of ammonium formate and 1 mL of formic acid. (Phase B: $[NH_4]$+[HCOO]- 2 mM, MeOH/ACN 50:50, 0.1% HCOOH, 1% H_2O), for 1 L of mobile phase B use 495 mL of methanol, 495 mL of acetonitrile, 10 mL of water, and add 126 mg of ammonium formate and 1 mL of formic acid.

The Q Exactive mass spectrometer was equipped with a Heated Electrospray Ionization Source (HESI-II) and was operated in the positive ionization mode. Parameters were optimized according to the methodologies from previous publications with some modifications [20-22], including a sheath gas flow rate of 53, an auxiliary gas flow rate of 14, and a sweep gas flow rate of 3 (manufacturer units). The spray voltage was set at 3kV, the capillary temperature was set to 269°C, the auxiliary gas heater temperature was set to 438°C, and the S-lens RF level was set to 55. The scan parameters were set as follows: Full MS scan with a resolution of 70,000, Automatic Gain Control (AGC) target 1e6, maximum Injection Time (IT) 100 ms, scan range 100 to 1000 m/z, and centroid spectrum data type.

For data acquisition, the TraceFinder 4.1 software from Thermo Scientific was used, and the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) was used to ensure data quality and manage the analytical processes. The compound database used was Synthetic Cannabinoids_2018, with modified data using reference standards.

Case Histories

A complete postmortem examination was performed on all cases including analysis and identification of samples of postmortem blood, and urine where a full survey was done to all samples and specimens received for each case in toxicology lab.

Systematic toxicological analysis was performed in each case, starting with a screening of urine and blood by means of immunoassay (Randox technology), as a routine analysis for the following substances: Benzodiazepines, barbiturates, tramadol, cannabinoids, amphetamine, methamphetamine, cocaine, opiates, tricyclic antidepressants, and synthetic cannabinoids. In addition to microline strip urine test for specific detection of synthetic cannabinoids. The positive results cases were confirmed by High-Resolution Accurate-Mass (HRAM) Orbitrap detection.

Three cases were identified for drugs abuse and one or more designer drugs (indazole-3-carboxamide family) were found and confirmed. The risk of these drugs is more when taken with other drugs which can produce unwanted increases in heart rate and blood pressure, which may leads to acute circulatory failure that followed to death according to pathologist investigation.

Case 1

A 35-years-old male was found as a corpse in desert after few days from disappearance. The pathologist collects the central blood from the body and bladder wash was also collected. Both blood and bladder wash give positive observation for natural/synthetic cannabinoids in screening test, which is later confirmed by LC/MS/MS as: 11-hydroxy-Δ^9-THC, THC-glucuronide, 5F-ADB, 5F-AB-PINACA, 5F-AKB48, and AKB48-N(4-hydroxypentyl) metabolites, in addition to benzodiazepines compounds that is found in blood.

Case 2

A male, in his 20 s, was found dead in his car in a parking. He was lying on the front of the driver seat, and he was noticed by the public. The police found small plastic envelope with white crystal, and cigarette butt in his car. The white crystal was examined and confirmed as a methamphetamine by LC/MS/MS and his biological samples (urine, blood) shows positive results for methamphetamine, amphetamine, 5F-ADB, FUB-AMB, MDMB-FUB, and AB-CHIMNACA, where two or more of SCs were found in each sample. The herbal residue in cigarette was analyzed as 5F-ADB, FUB-AMB, and AB-CHIMNACA.

Case 3

A 39-years-old male was found dead in car accident. He was known to heavily consume alcoholic beverages, and addict in smoking of drugs. The witnesses observed the car speeding in the roadway before collapsed. Ethyl alcohol was found in his blood, 5F-ADB, FUB-AMB, and AB-FUBINACA was found in his urine, and blood samples. In addition, the herbal blends give the same results as in his biological samples.

In all cases, 5F-ADB was detected which is a common synthetic cannabinoids in Kuwait, to be consumed by users. 5F-ADB (also known as 5F-MDMB-PINACA) and it was identified in last years as postmortem samples taken from an individual who had died after

Table 1: Observational data for three cases where synthetic cannabinoids, were implicated in the case history.

Case	Age	Gender	Case History	Symptoms	Laboratory Results
1	35	Male	Three friends were celebrating their time by taking drugs and narcotics in winter time were people go for camping in desert; one of them seems to have drugs overdose and have difficulty in breathing and died. His friends throw the body inside desert.	The body discovered after few days from disappearance. The investigators research for his last calls and they contact his friends. They said that he was long time addict to narcotics, and the day he died he was taking " chemical ".They said he was suffer from difficulty of breathing and lost consciousness, and pastaway. They throw him inside the desert.	5F-AB-PINACA 5F-ADB 5F-AKB-48 AKB-48-N (4-hydroxypentyl) metabolites THC Benzodiazepines
2	27	Male	Young male was found dead in his car in parking area. The public call the police	His friend confesses that they take methamphetamine, before smoking the synthetic marijuana.	Methamphetamine Amphetamine 5F-ADB FUB-AMB MDMB-FUBINACA AB-CHMINACA
3	39	Male	Middle age male found dead along the side of the road in a car accident. He was addict to smoked "synthetic marijuana". And also was known to heavily consume alcoholic beverages.	The witnesses observed a car speeding the roadway and collapsed. When ambulance arrived, he was pastaway.	Ethyl alcohol 5F-ADB AB-FUBINACA FUB-AMB

Table 2: Screening and confirmation results.

Case Number	Samples	Screening Results (ng/mL)	Confirmation Results
1	Bladder Wash	THC: + 65.42	11-hydroxy-Δ^9-THC THC-COOH-glucuronide
		ABPIN: + 25.18	5F-ADB, AKB-48 N(4-hydroxypentyl) metabolites
	Blood	THC: + 52.12	THC-COOH-glucuronide
		ABPIN: + 28.25	5F-ADB, 5F-AB-PINACA, 5F-AKB-48
		BENZ: + 10.87	Diazepam, Nordiazepam
2	Urine	ABPIN: +21.04	5F-ADB, FUB-AMB, MDMB-FUBINACA
		AMPH: + 13.35	Amphetamine
		MAMP: + 74.58	Methamphetamine
	Blood	ABPIN: +21.76	5F-ADB, AB-CHMINACA
		AMPH: + 30.06	Amphetamine
		MAMP: + 58.22	Methamphetamine
	Leaves in cigarette	-	5F-ADB, FUB-AMB, AB-CHMINACA
	White crystal reside	-	Methamphetamine
3	Urine	ABPIN: +28.05	5F-ADB, AB-FUBINACA
	Blood	ABPIN: +18.12	5F-ADB, FUB-AMB Ethyl alcohol: 0.21 g/100 mL
	Herbal blend material	-	5F-ADB, FUB-AMB, AB-FUBINACA

*ABPIN: Synthetic cannabinoids tests that is belong to carboxamide indazole group

using a product containing this substance. 5F-ADB is believed to be extremely potent based on the very low levels detected in many samples, and appears to be significantly more toxic than earlier synthetic cannabinoid drugs that had previously been sold [23-29].

The forensic pathologist assumed that the cause of death in all cases can be as a result from taken synthetic drugs with other narcotics and more examination done by toxicologists to determine the manner of death. Table 1 summarized the data for the three case histories.

Results

All cases in this study were investigated under authority of the general department of criminal evidence in Kuwait (Forensic Toxicology Lab) including performance of all toxicology tests, in addition to Pathology Lab in Forensic Medicine Department that is deal with the autopsy and samples provided. The three postmortem cases seemed to have been caused by synthetic designer drugs with a combination of other multi-drugs that can give adverse effects on human. All deaths associated with one or more of indazole-3-carboxamide group in both screening and confirmation analysis. These indazole carboxamide class synthetic cannabinoid have been particularly rampant, and exhibit severe toxic effects upon consumption due to their high binding affinity and potency at the cannabinoid receptors (CB_1 and CB_2) [30-37].

Table 2 summarizes the observational data for the three postmortem cases where synthetic cannabinoids and other drugs were implicated in the case history.

In all three postmortem cases shows a positive results of 5F-ADB in one or more of its biological samples, in addition to basic drugs such as benzodiazepines, methamphetamines, amphetamines, delta-9-tetrahydrocannabinol, and also ethyl alcohol which is detect by alcohol department (specific for alcoholic beverages), all these compounds were confirmed clearly by LC/MS/MS. All samples were analyzed in duplicate in both screening analysis through microline test and Randox, the positive results of screening are always verified by more specific methods such as analysis through mass spectrometer Benchtop LC-MS/MS system that combines quadruple precursor ion selection with High-Resolution, Accurate-Mass (HRAM) Orbitrap

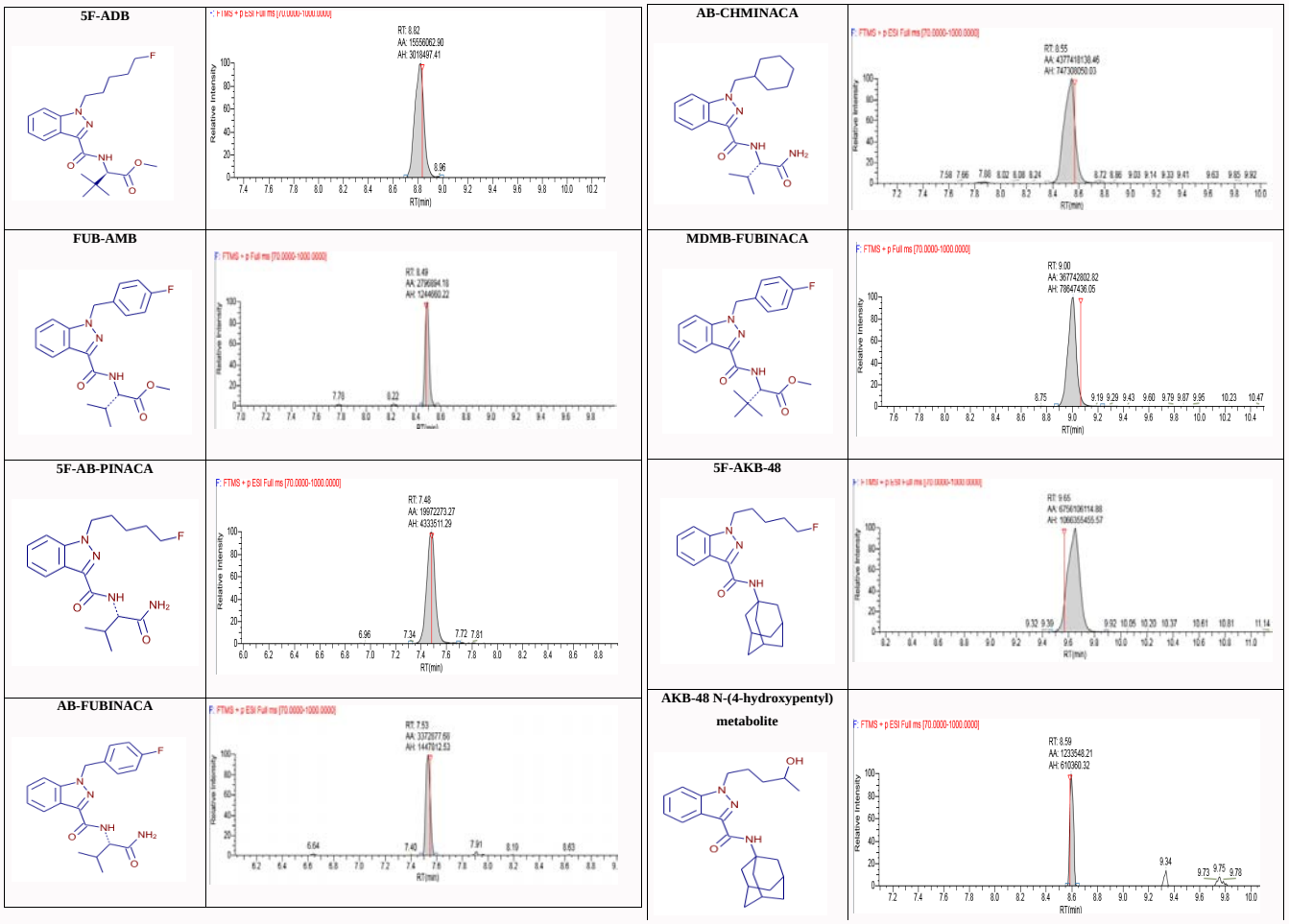
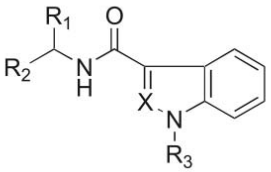


Figure 1: Chromatogram and retention time of carboxamide-3-indazole group.

Table 3: Chemical Structures of indazole-3-carboxamide group found in samples.



No.	Name	X	R_1	R_2	R_3
1	5F-ADB	N	$C(CH_3)_3$	$CO–OCH_3$	5-fluoropentyl
2	MDMB-FUBINACA	N	$C(CH_3)_3$	$CO–OCH_3$	4-fluorobenzyl
3	AB-CHMINACA	N	$CH(CH_3)_2$	$CO–NH_2$	cyclohexylmethyl
4	5F-AB-PINACA	N	$CH(CH_3)_2$	$CO–NH_2$	5-fluoropentyl
5	AB-FUBINACA	N	$CH(CH_3)_2$	$CO–NH_2$	4-fluorobenzyl
6	FUB-AMB	N	$CH(CH_3)_2$	$CO–OCH_3$	4-fluorobenzyl
7	5F-AKB-48	N	Admantyl	-	5-fluoropentyl

detection.

Further analysis was done for the white crystal found in case 2 that has been tested by taking a small amount of the residue and dissolve it in Ms-grade methanol and directly to be injected in both GC/MS and LC/MS/MS, where clearly two base peaks of 58 and 91 m/z shown in mass spectrometry in both libraries that is proceed all reference standards. In addition, analysis also done to the herbal leaves in cigarette. The leaves were examined under a stereomicroscope to examine their physical characteristics and noted that colorless microscopic crystals stuck to the surface of the dried leaves and plant materials. These crystals dissolved when rinsed with methanol, indicating that these compounds had been added to the dried leaves. The presence of crystal structures was used as an indication that synthetic cannabinoid powder may have been sprayed on the plant materials and dried leaves [17,18]. After physical examination of

Table 4: Method and MS/MS parameters utilized.

Synthetic Cannabinoids	Retention Time (min.)	Protonated Precursor Ion (*m/z*)	Collision Energy (*V*)	Product Ion (*m/z*)
5F-ADB	8.72	378.21	35	346, 318, 251, 233, 213, 177, 163, 145
AB-PINACA	7.97	331.21	35	314, 286, 215, 163, 145
5F-AB-PINACA	7.3	349.2	35	332, 304, 251, 233, 213,177, 163, 145, 69
AB-FUBINACA	7.63	369.17	35	352, 324, 271, 253, 109,83
AB-CHIMNACA	8.55	357.22	35	340, 312, 259, 241, 163, 145, 135, 117, 90
FUB-AMB	8.58	384.17	35	352, 324, 271, 253, 109, 83
MDMB-FUB	8.85	398.18	35	366, 338, 271, 253, 109, 83
5F-AKB-48 (5F-APINACA)	9.43	384.24	35	294, 215, 145, 135, 107, 93, 79

those materials, the samples were submitted to LC/MS/MS analysis in order to confirm the results and identify the types of SCs present, the various SCs combinations can be found, and with other types of illicit drugs mixed with the SCs. The types of SCs found in cigarette are: 5F-ADB, FUB-AMB, and AB-CHMINACA, in addition to these SCs is AB-FUBINACA that is found in herbal blend material in case 3.

The toxicology analysis revealed that the most common SCs in Kuwait is 5F-ADB, followed by FUB-AMB, AB-FUBINACA, and AB-CHMINACA and the majority mainly comes from indazole-3-carboxamide group, in addition to 5F-AKB-48 (5F-APINACA) in which R_1 is admantyl and R_3 is 5-fluoropentyl. Table 3 represents the SCs found in the postmortem cases.

The standards and samples were injected in High Resolution Accurate Mass (HRAM) Quadrupole-Orbitrap, and both retention time and products ions of samples were match those in standards and in literature survey. The data obtained can be summarized in Table 4 and Figure 1 where the most carboxamide indazole compounds found in both seized and biological samples.

Discussion

All of the synthetic cannabinoids tested in the present study of carboxamide indazole group, (5F-ADB, AB-PINACA, 5F-AB-PINACA, AB-FUBINACA, 5F-AMB, MDMB-FUBINACA and FUB-AMB) with high toxicity effects. These synthetic cannabinoids act directly at cannabinoid CB1 and CB2 receptors as does Δ^9-Tetrahydrocannabinol (Δ^9-THC) found in marijuana, but have different chemical structures unrelated to Δ^9-THC, different metabolism, and often greater toxicity that have been associated with significant adverse effects, including lethality.

For clinical evaluation, the situation is additionally complicated by the complex biotransformation of the synthetic cannabinoids, which may give rise to unpredictable pharmacodynamic effects, as the metabolites may act as agonists, inverse agonists or antagonists at the CB1 receptor. Therefore, the knowledge of the consumption pattern of an individual patient is a prerequisite for correct interpretation of analytical results. Further research activities are necessary to improve the interpretation of clinical and analytical findings [38-40].

Conclusion

Synthetic cannabinoids abuse is a significant public health problem, resulting in many emergency department visits and fatalities. Despite illicit drug scheduling by governments, novel SCs are consistently introduced. To counter this growing challenge, global collaboration is critical.

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