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GC-IR technique a rapid tool for detection of Tramadol in Forensic Confiscated Drugs and Biological Fluids

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Abstract: Tramadol is placed under Drug Controlled Substance Act, in India since 2018, owing to its extensive abuse as a narcotic substance recently. Subsequently, there is increased illegal trafficking across our nation for the drug tramadol. Forensic Science Laboratory, Delhi receives confiscated drugs and toxicological samples for chemical analysis to evaluate their precise composition enabling regulations from law enforcement agencies. Although considered a safe drug for its anti-inflammatory properties when used under medical guidance nevertheless, unintentional fatal tramadol intoxication due to its action on opioid receptors has been reported in several autopsy cases. Contraband tramadol is presently being examined using Gas Chromatography-Mass Spectrometer (GC-MS) and Fourier-transform infrared spectroscopy (FTIR) instrumental techniques in routine forensic examination. In the proposed study, an advanced sensitive technique Gas Chromatography Infrared spectroscopy (GC-IR) was explored as an alternative tool for rapid identification and confirmation. Standard Tramadol was used for method establishment and the method was subsequently applied to seized drug samples and Biological Fluids (blood, and urine samples). Findings of the study indicate that this method can be successfully applied to seized drugs and biological toxicological samples in the forensic examination for the drug Tramadol which was successfully detected by GC-IR. This new rapid analytical method will prove beneficial for the forensic community during routine examination of forensic samples with confirmation.

Keywords: Controlled substance, tramadol, trafficking, intoxication, forensic toxicology, GC-IR spectroscopy.

REVIEW PAPER

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INTRODUCTION

Tramadol was introduced to the world by German Scientist Dr. Kurt Flick in 1962, as a safe painkiller to handle extreme pain in life-threatening diseases (Scott L J *et al.*, 2000) [1]. Tramadol hydrochloride, chemically known as (±)-trans-2-[(dimethylamino) methyl]-1-(3-methoxyphenyl) cyclohexanol, is pharmacologically responsible for analgesic efficacy, even though its analgesic effect is much less potent than morphine (Duehmke R M *et al.*, 2017, Baltazar-M G, 2019) [2, 3]. Belonging to the Opioid analgesic class, its action is exerted through both opioid and non-opioid pathways, involving μ-opioid receptor activation as well as inhibition of serotonin and noradrenaline reuptake (Bravo L, 2017, Raffa RB, 2017) [4, 5] and thus, carries much dependence perspective on long term use (Dayer P) [6]. The mechanism of serotonin and noradrenaline reuptake is similar to the action of some antidepressants, and preclinical studies have confirmed an antidepressant effect of tramadol (Bravo L, 2017) [4]. Because of dual mechanism of action, this atypical opioid is popular among abusers and its impact has been demonstrated in many studies (Tjaderborn M *et al.*, 2007, Pothiawala S *et al.*, 2007, Saleh SM *et al.*, 2016, Bachs L *et al.*, 2009, Clarkson JE *et al.*, 2004, "Isis drug", Skipper G *et al.*, 2004, Ojha R *et al.*, 2010, Cicero T *et al.*, 1999, Sarkar S *et al.*, 2012) [7-16]. Generally, drug abuse starts as counter-prescription drug (Ferrari F *et al.*, 2020) [17] later being carried as addiction leading to life threatening conditions due to intoxication as reported (Sarkar S *et al.*, 2012, Ferrari F *et al.*, 2020, Musshoff F *et al.*, 2001, Prakash J *et al.*, 2010) [16-19] creating a reason for concern regarding addiction. The most frequently observed side effects of tramadol are drowsiness, dizziness, sweating, fatigue, nausea, vomiting, headache, and dry mouth depending on individuals widely acted dose requirements, and minimum effective blood level. The relationship between drug concentration and drug effect in living individuals is not directly proportional, one major

reason is the large inter-individual differences in drug response (Trescot A M *et al.,* 2014) [20]. Some severe complications in case of intoxication result in seizures, hepatic, failure, and respiratory distress (Backer B De *et al.,* 2010) [21].

India's sprawling pharmaceutical industry fueled by cheap generics and incentive money-making made the country more vulnerable to drug trafficking, addiction, and multiple additional associated crimes. International trafficking of tramadol came under enhanced surveillance; and its smuggling through India was reported by Narcotics Control Bureau, India in March 2018. The Indian Government later regulated tramadol in 2018 under Schedule 4/(IV) of the Drug Controlled Substance Act, NDPS Act, 1985, to curb drug addiction and drug dependence (Srivastava N *et al.,* 2018) [22]. The growing population of drug abusers and its associated problems necessitate forensic scientists to develop new approaches for rapid drug detection and confirmation to ascertain the contraband presence in seized samples. An alternative technique Gas Chromatography –Infrared Spectroscopy (GC-IR) was explored by a forensic scientist in Delhi (India) for the first time for the identification of Tramadol in seized drugs and biological fluids. GC-IR, a coupled technique comprising GC and FT-IR (Fourier-transform infrared spectroscopy), is characterized by quick scanning to obtain the IR spectra of peaks eluting from GC capillary columns. This technique combines the chemical separation power of GC and the chemical identification power of IR to provide improved results owing to unaltered characteristics and spectroscopic features of compounds using this cryogenic sample deposition over vapor phase technology (Praisler M *et al.,* 2001, Praisler M *et al.,* 2000, Salerno TMG *et al.,* 2020) [23-25] that will prove beneficial for the forensic community. Forensic drug analyst is in search of different techniques for rapid and accurate analysis of the substance of abuse as there is the relative increased speed of entry and exit of crime cases which make it incredibly difficult to respond on the relevant time scale.

METHODS

Chemicals Required

Standard Tramadol was obtained from United Nations on Drugs and Crime. Chloroform, Diethyl ether, Methanol, Ammonia, Sodium tungstate, Sulphuric Acid, and Anhydrous sodium sulfate were of Analytical grade purchased from Sigma. Standard solutions and case samples were stored at -4^{o}C when not in use.

Sample preparation

Case samples

Confiscated drug samples dissolved in methanol and were run on GC-IR and toxicological case samples were also analyzed by the same methodology.

Blood extraction procedure

Blood (5 ml) is mixed with 5 ml of 10% sodium tungstate solution and 1 ml of 1N Sulphuric acid and heated at 60^{o} C for one minute followed by vortex for two minutes to obtain a clear separation. The clear filtrate is collected and transferred into a separating funnel and made basic by adding a drop of liquid Ammonia. The filtrate is then extracted thrice with a 5 ml of diethyl ether and Chloroform (1:1) together. The organic layer is passed through anhydrous sodium sulfate and the solvent is concentrated to obtain the final sample for analysis in GC-IR.

Urine extraction procedure

2 drops of liquid Ammonia are added to 5 ml of urine to adjust the pH to Basic. The extraction process is similar to blood extraction.

Standard Tramadol samples were prepared in six concentration ranges starting from 25µg/ml to 1000µg/ml (25 ppm, 50 ppm, 100 ppm, 250 ppm, 500 ppm, and 1000 ppm). Blanks were run in between samples. All samples and standards were prepared by dissolving in HPLC-grade methanol for further analysis in instrument GC-IR. These different concentrations of standards were run on GCIR to measure (a) Accuracy (b) Specificity (c) Repeatability and (d) Sensitivity for method designed. Further, Confiscated drug samples, seized blood, and urine samples were used as application for the method developed.

Instrument

GC-IR studies were carried out on Dani Master AS, Gas Chromatography (GC) coupled with Autosampler connected to DiscovIR (FT-IR Unit) as shown in Fig 2. In this Mercury-Cadmium-Tellurium (MCT) cryogenically cooled IR detector was used to obtain spectra in the range of 4000 to 700 cm^{-1} with a resolution of 4 cm^{-1}. The interface connected by the transfer line between GC and IR has a temperature of 280°C. The ZnSe disc in the detector was set with a rotation speed of 3mm/min where the samples were cooled at -40°C and maintained by liquid nitrogen for detection. GC was operated in split-less mode with Helium as carrier gas and DN 5 column (15m x 0.25mm ID x 0.25 µm film thickness). The temperature was programmed at 90°C to reach 290C at the rate of 34°C/min for 5 min to cover the total run time in 12 minutes.

Data Acquisition and Processing

GC-IR data acquisition and processing are performed on Gram's software. Compounds were identified through the library search program from the available inbuilt library. The results were compared with the inbuilt software shown in points ranging from 0-1. Instrument operations and data analysis were conducted using workbooks designed in Grams software by Dani Instruments.

RESULTS

Six concentrations of Certified Reference Material tramadol (ranging from 25 ppm to 1000 ppm) were run on GC-IR and the resultant graphs are shown in Fig 1B. Identification of sample was done with 98% library matching for 1000 ppm as shown in Fig 1A and confirmation with the presence of tramadol by characteristics peaks as shown in Fig 1D with wave number 2996, 2938, 2855, 2828, 2782, 1608, 1601, 1583, 1483, 1462, 1448, 1429, 1317, 1288, 1269, 1251, 1166, 1046, 991, 977, 783 cm^{-1}. The high discrimination power of IR was appreciated from the comparison of stacked IR spectra of the different concentration ranges of standard and samples, especially in complex fingerprint regions illuminating best match with the library Fig 1B. The detection limit of tramadol by these parameters was found to be 0.0001 ng in standard. Repeatability of samples was established with 100 ppm sample run in triplicate and accurate results with identified peaks were obtained as depicted in Fig 1C. Sensitivity was evaluated by determining the limit of detection by running different concentrations of the standard drug where 50 ppm standard or below was not detected. Inter-day and intra-day accuracy and precision were assessed by comparing data run on different days during the validation process to determine the target drug as shown in Fig 1. Each time the drug was separated and identified by the GC-IR method adopted as mentioned in instrumental parameters. Thus, the accuracy, precision, specificity, and repeatability of standard tramadol was successfully conducted.

Based on the above results, seized drug samples and biological fluids (blood and urine) were also run and results demonstrated excellent corroboration with standards as shown in Fig. 2A, 2B, and 2C.

This method of detected and identified of Tramadol was found equally suitable in biological specimens/fluids collected as part of forensically relevant casework including toxicological examination, a part of medico-legal death investigations.

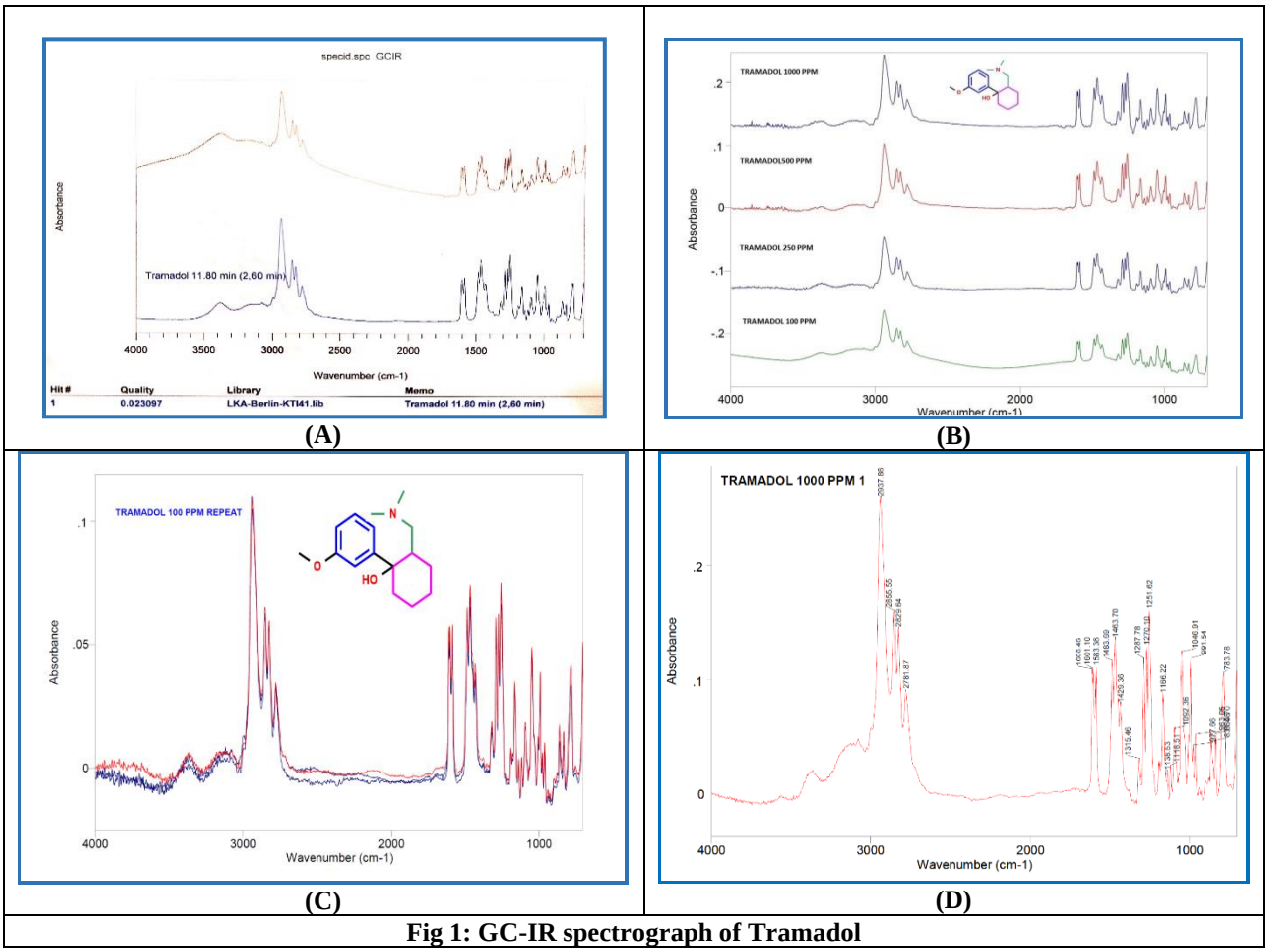

Fig 1: GC-IR spectrograph of Tramadol

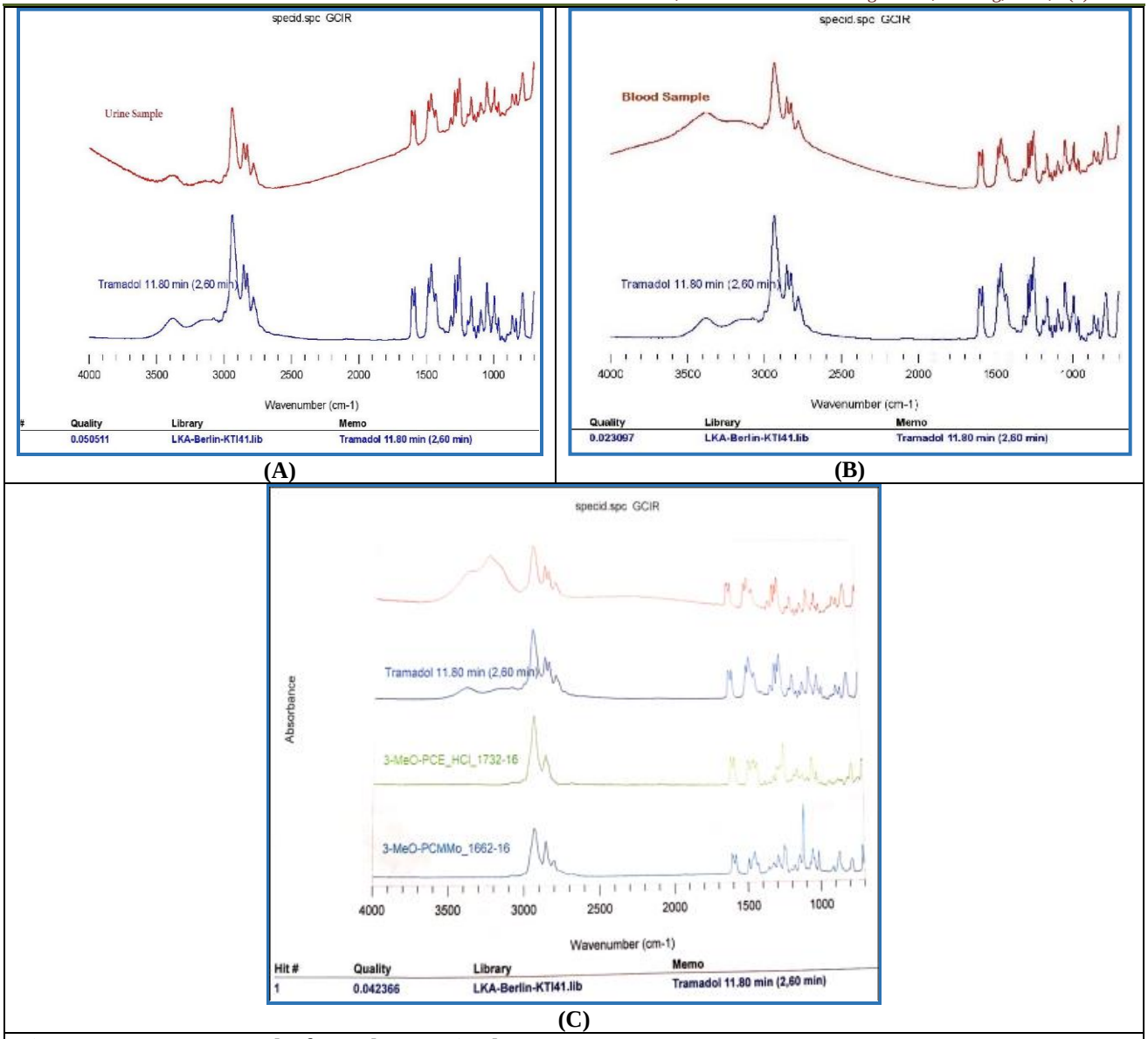

Fig 2: GC-IR spectrograph of samples examined

DISCUSSION

Tramadol is a widely abused illegally trafficked drug in India reason being its easy accessibility as over counter drug and its low cost. Also, the geographical location of India has been well utilized to dupe law enforcement agencies by drug traffickers to enhance their trading and sale. Illegal trafficking and seizures of tramadol have been documented by the Directorate of Revenue Intelligence in its annual report 2019-2020 and noted by scientists abroad (DRI Report 2020, Progler Y 2010, Clarot F *et al.*, 2003) [26-29]. The Worldwide trend of Tramadol use observed by World Health Organization was more than double from the year 2001-2003 to 2011-2013 (World Health Organization 2018) [30]. Unlike other opioids, tramadol flowed freely around the world, originating from India, unrestricted by international controls which made United Nations identify tramadol as a schedule drug owing to its abuse and ill effects on society. Thus, the scientific identification of tramadol plays a crucial role in the criminal justice system.

Successful instrumental determination of tramadol has been a focus of many studies over the past few years by GC-MS (Gas Chromatography-Mass spectrometry) and FT-IR (Yilmaz B *et al.*, 2015, Arjunan V *et al.*, 2014, Hadidi K *et al.*, 2003) [31-33]. FT-IR and GC-MS are still conventional methods for analysis demonstrating promising results for the identification of unknown drugs in forensic samples. Forensic examination of controlled substances and biological exhibits have also been done by other hyphenated techniques like LC-MS/MS (Liquid chromatography-mass spectrometry), UPLC (Ultra-performance liquid chromatography) (Luginbühl M *et al.*, 2020, Sabbah AI *et al.*, 2015, Goeringer KE *et al.*, 1997, El-Sayed AA *et al.*, 2019, Mahdy T *et al.*, 2012, Belal T *et al.*, 2009) [34-39]. Most of these instruments require a standard of Drug during the examination of

forensic case exhibits. This limitation is appropriately overcome by GC-IR, where the forensic case samples can be also be identified without a standard drug with confirmation, as it has an IR detector. The Scientific Working Group for Analysis of Seized Drugs endorses minimum standards for forensic identification of frequently seized drugs. GC-MS results of case exhibits are the probable matching of the database whereas GC-IR gives the confirmed fingerprint for identification. Hence, the complete analysis of case exhibits requires multiple uncorrelated techniques for drug detection and confirmation. Hyphenated techniques like GC-IR is considered separate technique where the results of each method are used for identification and detection. Herein, the GCIR initiative has been undertaken in authentic forensic specimens where urine and blood are specimens of choice in both postmortem and toxicology to establish drug exposure. Therefore, the authors were convinced to apply GC-IR as a tool for considering the universal forensic application approach (Wyman JF 2012, Smith MP *et al.*, 2016, Skopp G *et al.*, 2010, Kennedy MC 2010) [40-43]. To utilize the results in the court of law, specificity, accuracy, and precision during drug analysis are important measures. GC-IR with solid phase IR spectra was evaluated for its analytical specificity for tramadol (Fig-1A) and successfully demonstrated separation, and repeatability through consistent retention times that matched with reference spectra of tramadol from instrument library. The method employed demonstrated spectra reproducibility in certified reference material where all spectra fell within the interpretation range (above 50 ppm) and were well distinguishable (Fig-1B). The method was further also applied to case samples and was found suitable (Fig-2A and 2C). The effective use of GC-IR is due to the interface between separation and identification making it a better analytical technique in comparison to FT-IR. Therefore, the developed method for analyzing tramadol with GC-IR can easily complement the forensic technique GC-MS. The observations together with discussion advocate use of GC-IR as an additional technique in routine testing in forensic laboratories.

CONCLUSION

In this study, a new rapid qualitative analysis method for the detection and identification of tramadol in seized contraband and biological samples was developed using GC-IR with a total run time below 12 minutes. This methodology with reproducible, reliable, and sensitive results has become a valuable resource of our laboratory for the chemical analysis of forensic exhibits. The cryogenic solid phase deposition on the ZnSe disk enabled enhanced resolution and sensitivity of the technique, scaling down the limit of detection to the ng level. This study explored the examination of tramadol in blood and urine which would be highly valuable in intoxication cases, where matrix interferences hinder accurate identification. The effectiveness of solid phase GC-IR is well demonstrated as an alternative tool to the widespread MS-based approach. It provides complementary information for the confident identification of drugs in forensic samples.

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