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REVIEW

Analytical Strategies for Identifying DFSA Drugs Across Biological and Non-Biological Matrices: A Systematic Review

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Drug-facilitated sexual assault (DFSA) involves the covert administration of psychoactive substances commonly benzodiazepines, γ-hydroxybutyrate (GHB), ketamine and related sedatives to incapacitate victims and impair resistance and memory. These drugs are often present at trace levels, rapidly metabolised and may be detected long after the assault, making their analytical identification both challenging and crucial in legal investigations. The present systematic review reports recent advances in the detection, separation and quantification of DFSA-related drugs across diverse matrices including biological fluids (blood, urine, plasma, breastmilk), beverages, dried blood spots and surface residues. Analytical strategies evaluated chromatographic, spectrometric, spectroscopic, electrochemical and microextraction-based methods, with strong emphasis on hyphenated approaches such as GC-MS, LC-MS/MS, UHPLC-HRMS, Raman spectroscopy, FTIR and voltammetry-based portable sensing platforms. The findings indicate that emerging analytical approaches, particularly dispersive liquid-liquid microextraction (DLLME), microextraction by packed sorbent (MEPS), microwave-assisted extraction, electrochemical paper-based devices, and dried blood spot (DBS) workflows, provide improved sensitivity, reduced sample volume requirements and rapid screening capability for both laboratory-based and field forensic investigations. The review further demonstrates that no individual analytical method is equally effective for all biological matrices; however, the combination of advanced extraction strategies with high-resolution analytical instrumentation substantially enhances the detection of low-concentration DFSA compounds. These observations highlight the growing importance of modern analytical technologies in enabling accurate and timely identification of DFSA agents, thereby supporting more reliable medico-legal investigations in cases of sexual assault.

Keywords: Date-rape drugs, Spectroscopy, Biological fluids, Beverages, Benzodiazepines.

INTRODUCTION

Rape drugs, often known as date rape drugs, are drugs which are used to facilitate sexual assault by knocking the victim out or making it harder for them to resist [1,2]. These substances are usually introduced to a victim's drink and upon consuming the drug, the intended victim can become unconscious and then wake up with no memory of the events that occurred during the period of attack meaning the attack can never be proved or stopped [3]. This is the reason, drug facilitated sexual assault are emerging as an increasing concern to the health community [4]. The common drugs involved in drug facilitated sexual assault are rohypnol (flunitrazepam), γ-hydroxybutyric acid (GHB) and ketamine (Table-1) [5,6]. The toxicological analysis to detect these drugs can be performed on several matrices such as blood, plasma, urine and breast milk [7]. Detection of these drugs from the biological matrices is challenging as they are highly potent, requires only administration of small doses, which gets rapidly eliminated from the body, leading to low concentrations in biological fluids [8]. Furthermore, the drugs are no longer present in the preliminary test samples including blood and urine by the time of the complaint since the victims often delay reporting the incident [9]. As a result, confirming the use of these drugs remains a significant challenge in forensic toxicology [10].

Blood is a very intricate medium which contains a host of components that could potentially affect the way drugs are extracted and quantified [11]. The blood matrix impact on drug ionisation and detection is usually insignificant and differs. Hence, method optimisation is crucial to ensure the correct

TABLE-1
LIST OF DFSA DRUGS AND THEIR METABOLISM, BRAIN EFFECTS, CLINICAL USE & ONSET OF ACTION

Drug	Onset	Metabolites	Clinical usage	Neural recognition sites
Flunitrazepam	15-20 min	3-Hydroxyflunitrazepam, 7-aminoflunitrazepam	Anticonvulsant, amnestic, sedative	GABA receptor
GHB	15-20 min	γ-Butyrolactone, 1,4-butanediol	Narcolepsy, sedative	GABA-B receptors
Ketamine	5-20 min	Norketamine, dehydroxynorketamine, hydroxynorketamine	Anesthetic, sedative	NMDA receptor, non-NMDA glutamate receptor, opioid receptors,
Diazepam	30 min to 1 h	Desmethyldiazepam	Anxiety disorders, seizures, Sedatives	GABA-A receptor complex

outcome of the given problem [12,13]. Furthermore, the blood detection window often closes by the time victim regain consciousness, urine tests are generally preferred as they remain active for a longer period while blood tests are most effective if conducted within 4 h of the intake of the drugs [14]. Urine is frequently used owing to the presence of metabolites and relatively non-invasive method of sample collection [15]. In accord with the above-said statements, it is possible to use such techniques as ultrasound-assisted liquid-liquid microextraction (UA-LDS-DLLME) and paper-supported polystyrene membranes for the successful extraction of benzodiazepines and date rape drugs from urine matrix [16,17].

Plasma is a fluid which is a good solvent that is important in studying medication uptake and distribution, while serum is acquired from the blood once the coagulant forms and comprises proteins and other chemicals that interfere with the evaluation of the drug [18]. As for the exact determination of drugs in plasma, there is the need for the highest extraction efficiency as offered by techniques such as DL-LME and SPE [19,20]. In addition, Furugen *et al.* [21] described the procedures for sample preparation, extraction and analysis used to isolate condition-specific pharmaceuticals from breast milk, clearly outlining the steps required for the identification and quantification of metabolites. Following this, they also validated their method through the determination of protein binding of diazepines in plasma, emphasising calibration, precision, accuracy and protein binding as critical performance parameters demonstrated in their study. The study confirmed that the quantification was exact during the tests and it was revealed that plasma is used to know the drug transfer process into the breast milk. Furthermore, there is a new method that uses a solid-phase extraction (SPE) and liquid chromatography tandem mass spectrometry to detect drugs like sedative hypnotic from the plasma. The method also provides a well-structured linear model and reliability for the detection of these drugs, thus allowing its use in clinical and forensic toxicology [22].

Methodology

Protocol: A predefined and exhaustive review protocol was not prepared prior to conducting the study. However, the methodological framework adopted for the review was aligned with the preferred reporting items for systematic reviews and *meta*-analysis protocols (PRISMA-P) guidelines to ensure methodological rigor and transparency throughout the review process.

To adequately address the objectives of the review, certain elements of the protocol were adapted and customised in accordance with the scope and thematic requirements of this study. All reporting components, including the procedural flow, eligibility criteria, search strategies, study selection process and data extraction plan, were structured based on the preferred reporting items for systematic reviews and *meta*-analyses (PRISMA) standards (Fig. 1). The adapted protocol ensured a systematic and unbiased approach to identifying, screening, selecting and synthesizing the available literature relevant to the research question.

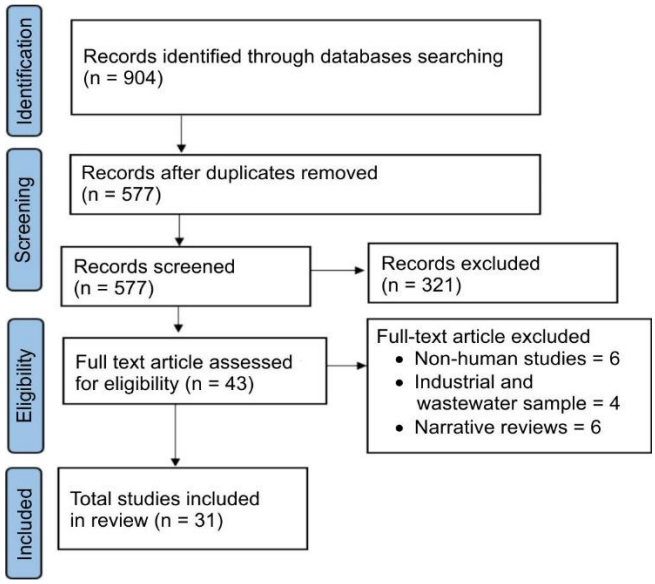

Fig. 1. Flowchart: Selection of sources

Data sources: The search for relevant evidence sources was conducted using an exponential electronic search approach, primarily within the ScienceDirect database and pubmed. No additional databases were consulted for supplementary or grey literature, as the focus of the review was to synthesize methodologically robust and peer-reviewed scholarly publications.

The search strategy consisted of multiple combinations of medical subject headings (MeSH) terms and Boolean operators to capture various conceptual expressions related to drug-facilitated sexual assault (DFSA). Keywords and search phrases included but were not limited to: date-rape drugs, sedatives, benzodiazepines, diazepam, clonazepam, drug-facilitated sexual assault, rohypnol, extraction, gas chromatography, high-performance liquid chromatography, attenuated total reflectance, Fourier transform infrared spectroscopy, organic extraction, efficiency, detection and identification in different matrices.

Synonyms and abbreviations representing each of the above concepts were incorporated using the Boolean operators "OR" and "AND" to ensure comprehensive retrieval of all potentially relevant sources within the target database. The search framework was refined iteratively and tailored to the functional features of the database interface to maximize output and maintain a focus on the subject area.

Eligibility criteria: Being a systematic review, the study employed broad criteria in order to ensure that only the date rape drugs could be isolated and identified from different matrixes. Some of the methods used in the analysis were detection and characterisation of different drug components in biological fluids and different beverages, which helped in retrieving sources that were relevant to the objectives set in the review that sought to identify the different types of sedatives, the quantities of each used and measures applied to isolate each of them. Articles published in languages other than English were excluded to avoid potential translation inaccuracies and minimise the risk of misinterpretation of the reported findings. Moreover, narrative review articles, conference papers and book chapters were also not included in the review as a way of only focusing on high level primary evidence.

Source selection: The final sources were subjected to two rounds of screening. The first step was the elimination of duplicate articles, which were executed with the help of free online software but then rechecked manually. When the duplicated sources were eliminated from the search, the titles, abstracts and keywords of the sources were scanned in order to select the ones that referred to the two major ideas and those that were not in English. In the second phase, the abstracts of sources searched with title were scanned to include those relevant for the review topic and to determine the methodology used to include whether or not drug analysis was done. Another advantage that the screening of abstracts allowed the reviewers was the ability to spot more articles whose methodological approaches would fit the purpose and selection criteria of the review.

The pilot database search produced a total of 904 articles and the first cut through the selection process of eliminating duplicates and papers published in languages other than English, as well as papers related to other fields. In the second phase, a total of 45 sources underwent full-text analysis for relevance and methodological rigor, resulting in the exclusion of 16 sources, six of which were narrative reviews, six studies on only non-human matrices and four in industrial and wastewater samples. Screening of the reference sections of these included articles revealed further additional sources and upon their inclusion the systematic review comprised of 31 articles.

Data extraction and charting: For data extraction, a structured data extraction chart was prepared and followed while extracting data from sources. To be more concrete, the data described the most essential information concerning each of the presented studies: the publication year, the aims of the study used instrumentation, methods and the identified drug, the key findings. These characteristics were also applicable to the process of synthesizing the results obtained in all the studies included in the analysis.

The main findings from the included studies are summarised in tabular form, followed by the type of studies, analytical methods, types of drugs in various biological fluids and beverages, sample preparations and prospects (Table-2). The results synthesis also entailed the classification of the goals and a consideration of the methodological approaches employed by the researchers.

TABLE-2
OVERVIEW OF DFSA-RELATED DRUGS IDENTIFIED IN BIOLOGICAL AND NON-BIOLOGICAL MATRICES, ANALYTICAL APPROACHES AND KEY PERFORMANCE OUTCOMES

Sample/biological matrix	Target drug(s) detected	Analytical strategy (extraction + detection)	Key outcomes/performance summary	Ref.
Urine	Designer benzodiazepines	Sonication + LLE → GC-QQQ-MS	Increased solvent dispersion and extraction efficiency.	[16]
Blood	Ketamine, flunitrazepam, cocaine	DI-SPME → LC-MS (ESI + TOF)	High recovery (94.6-106%); acceptable matrix effects (81.7-116.5%).	[23]
Whole blood	Several DFSA-related drugs	DLLME (chloroform + methanol) → UHPLC-QQQ-MS	LOQ 0.05–2 ng/mL; LOD 0.2–10 ng/mL; validated on 50 blood samples.	[24]
Urine	18 DFSA-related drugs	LLE + HPLC → LC-MS/MS	High-sensitivity quantification with reduced analysis time; ZPCA most frequent.	[25]
Breastmilk, plasma	Alprazolam, bromazepam, clonazepam, CM7116 (metabolite)	LLE → LC-MS/MS	Accurate benzodiazepine quantification; LLOQ 0.25–1.0 ng/mL.	[21]
DBS (blood)	Benzodiazepines, ketamine, stimulants	MAE + DBS → LC-MS	High extraction efficiency; validated for forensic DFSA.	[13]
Plasma	Clonazepam	UA-EME (hollow fiber) → CE-DAD	Extraction yield rose to 58%; LOD 3 ng/mL; high throughput.	[26]
Serum	Designer benzodiazepines	LLE → NACE-MS/MS	High selectivity, accuracy and linearity; improved resolution using coated capillary.	[27]
Urine	13 Benzodiazepines & metabolites	Enzymatic hydrolysis + LC-HRMS	Broad sample identification; reliable qualitative screening.	[28]
Sweat, saliva, breath, hair	Metizolam	ALT matrices + UHPLC-Q-TOF-MS/NMR	Demonstrated efficiency of alternative matrices; challenges due to nonauthorised status.	[29]
Dried blood spots	Benzodiazepines & metabolites	SPE → LC-MS/MS	DBS stable for 3 months; <20% deviation *vs.* whole blood.	[30]

Plasma	5 DBZDs & 3 Z-hypnotics	Protein precipitation + MEPS → HPLC	>90% recovery; <5% RSD; compliant LOD/LOQ.	[31]
Blood, beverages	Ketamine	MAE + DBS → CE-TOF-MS	High sensitivity; eco-friendly; suitable for rapid toxicology.	[23]
Plasma	Seven benzodiazepines	UA-DLLME → UPLC-PDA	Recovery 71-102%; fast and clinically usable.	[20]
Urine	Flunitrazolam & metabolites	LC-MS/MS + NMR	7-amino-FNTZ major metabolite; detectable up to 37 h post-dose.	[32]
Serum	Alprazolam, clonazepam, diazepam	HPLC-DAD	High precision and accuracy.	[33]
Aqueous samples	Phthalate esters	AALLME → GC-FID	Best performance at 8 extraction cycles; excellent detection limit.	[34]
Blood	Benzodiazepines, ketamine, GHB	MAE → UHPLC + TOF-MS	Accurate DFSA screening at low concentration.	[12]
Commercial beverages (Italy)	GHB	SPE → GC–MS	Differentiated endogenous *vs.* spiked GHB in beverages; highly sensitive method for forensic DFSA investigation.	[35]
Spiked alcoholic beverages	Diazepam, alprazolam, bromazepam, clonazepam, cloxazolam	LLE → Low-temperature Paper Spray–MS	Sensitive identification of 5 benzodiazepines in beverages; robust despite matrix effects; suitable for DFSA casework.	[36]
Grappa (diluted)	Chlordiazepoxide, lorazepam, diazepam, oxazepam, medazepam	Off-line MEPS → UHPLC-PDA & micro-ECD	High linearity & sensitivity; low solvent use; validated rapid identification of benzodiazepines in beverages.	[37]
Alcoholic beverages	Flunitrazepam	Modified screen-printed electrochemical sensor → Cyclic voltammetry	Rapid onsite detection; high selectivity & repeatability; potential deployment in DFSA prevention.	[38]
Alcoholic & soft drinks	Flunitrazepam	Screen-Printed electrochemical cells → Voltammetry	Lab-free electrochemical sensor; LOD 0.7 μM; excellent reproducibility and sensitivity for DFSA screening.	[39]
Beverages	Midazolam (also flunitrazepam, ketamine, GHB evaluated)	ePAD → DPV/SWV; validation *via* HPLC	Portable device; LOD 2.0 mg/L; fast onsite screening for sedatives relevant to DFSA.	[40]
Alcoholic beverages	Flunitrazepam	Raman spectroscopy → Portable FSX	Non-destructive, fast detection *via* direct spectra; strong application in DFSA investigations.	[41]
Alcoholic beverages	Flunitrazepam; also demonstrated GHB & ketamine	Electrochemical potentiostat + screen-printed sensor	Rapid onsite detection without sample pretreatment; suitable for screening spiked beverages.	[38]
Beverages/biological fluids	GHB and analogues (GBL)	LLE → FTIR (validated *vs.* LC-MS)	Excellent agreement with LC-MS (slope 1.025); GBL >90% stable at 5 °C for 2 weeks; temperature-dependent degradation.	[42]
Spiked beverages	Diazepam, flunitrazepam, temazepam	GC–MS (validated method)	Drugs detectable up to 25 days post-spiking; highlighted importance of drink matrix variability in DFSA evidence.	[43]
Beverage samples	Multiple DFSA drugs depending on test reagent	SERS drug-detection coasters + HPLC	High specificity for drug-free drinks; practical constraints include equipment cost and limited beverage range.	[44]
Residues on beverage surfaces	Benzodiazepines incl. triazolam	Surface swabbing + DLLME → HPLC-HRMS/MS	Detected trace BZD residues on drink surfaces even without biological samples; valuable for DFSA where bodily evidence missing.	[45]

The studies incorporated in this review involved the execution of cross-sectional examinations between 2013 and 2024. The most investigated topic was the isolation of date rape drug in different matrices, using an explorative approach to determine what types, concentrations and extraction techniques of date rape drugs and their presence found in different matrices [29,41]. As shown in Table-1, the studies on the subject can be categorised into five groups based on the research focus. Studies that focused on drug instrumentations highlighted the usage of each instrument in different specifications. The studies that focused on the methodological approaches highlighted the methods used in collecting and processing different biological fluids and beverages for further analysis to find out different drugs specifically quantification of benzodiazepines [28]. The methodologies used for drug quantification are an important aspect of the subject as they appear to account for a significant number of inconsistencies in findings. Standardisation of quantification techniques is a priority for research on the subject, the success of which has implications for the potential future use of benzodiazepines as a date-rape drug. The second category of studies focused on the types of drug used as Date-rape drug. The most reported drug, however, is

used consistently, regardless of the legal consequences or the onset of action of that specific drug. These include flunitrazepam, ketamine, GHB, metabolites of benzodiazepines. In addition, the electrochemical method used to identify drugs in spiked beverages showed that it does not require pretreatment or adjustment of the electrodes during the analysis, which enhanced the confirmation that the method is successful in identifying drugs [38].

Date rape drugs (DRDs) are difficult to extract and identify due to the variety of matrices, including blood, urine, solid surfaces and alcoholic and non-alcoholic beverages [46]. The stability of benzodiazepines and other DRDs can be impacted by some matrices, particularly sugary drinks or complex formulations like J2O [43,47]. As a result, techniques must be optimised by adjusting solvent volumes, pH and temperature to ensure accurate findings. As the results, in the perspective of the extraction efficiency of the mixed samples, it has been shown that the use of SPME as well as UA-DLLME as the suitable techniques for the separation of DRDs from the complex matrices. These techniques are of high sensitivity and selectivity where they are applied in combination with spectrometric and chromatographic procedures for identification of pharmaceuticals at the pg or ng level. The identification of chemicals that are thermally labile is further improved by derivatisation procedures and one of which is BSTFA-TCMS in GC-MS. Moreover, the analysis of complex multivariate data has been also improved by chemometric methods including principal component analysis (PCA) and partial least squares (PLS regression). Such techniques are useful for the identification of DRDs in combinations or in traces and this is very beneficial for forensic analysis [40].

Furthermore, in DFSA, the limit of detection and recovery are important parameters for reliable results as drugs often present at ultra-trace levels due to delayed reporting and rapid metabolism. LC-MS/MS provides superior sensitivity with lower limit of detection in low range for benzodiazepines and other related compounds in urine and blood. In contrast, GC-MS is highly reliable but may exhibit higher detection limits for various polar or thermally labile compounds until derivatisation is used. LC-MS/MS gives high and reproducible recovery with SPE for a wide range of DFSA drugs. GC-MS and HRMS may give variable recovery depending on the physiochemical properties of the drug.

Selection of analytical methods for DFSA cases based on reporting time and sample type: The choice of analytical method is strongly influenced by the time elapsed between the incident and sample collection. In early reporting cases (typically within 12-24 h), the primary objective is rapid and sensitive detection of parent drugs and short-lived metabolites in blood and urine. In such scenarios, targeted LC-MS/MS methods are often preferred due to their high sensitivity, excellent selectivity and ability to quantify a broad range of commonly encountered DFSA agents including benzodiazepines, Z-drugs and sedative-hypnotics. Blood samples are particularly valuable in this window, as they reflect recent exposure and allow for more direct toxicological interpretation.

In contrast, delayed reporting cases employs a higher analytical challenge due to the frequent metabolism and elimination of the drugs. In such type of cases, analysis of urine extends the window of detection and is often supplemented by hair analysis for contemplative detection over days to months of the drug exposure. For such type of cases, high-resolution mass spectrometry (HRMS) becomes significantly important as it permits untargeted or data-independent screening for an extensive variety of known and emerging drugs, even when the specific drug is undetermined during the analysis time. For the cases which involves suspected undetermined drugs or polysubstance exposure, a combined strategy is often required. Broad-spectrum screening using HRMS can be done initially which can be followed by confirmatory and quantitative analysis using LC-MS/MS. GC-MS may still be applied in specific situations where volatile or thermally stable compounds are suspected or where spectral library matching provides an advantage.

Conclusions

The use of spectroscopy combined with chemometrics has greatly improved the detection and extraction of date rape drugs (DRDs) such as benzodiazepines, clobazam, clonazolam, deschloroetizolam, diclazepam, estazolam, etizolam, flubromazepam, flubromazolam, flunitrazolam, 3-hydroxyflubromazepam, 3-hydroxyphenazepam, ketazolam, meclonazepam, metizolam, nifoxipam and pyrazolam, γ-hydroxybutyrate (GHB), γ-butyrolactone (GBL), alprazolam, bromazepam, clonazepam, diazepam, zolpidem, temazepam, flunitrazepam and ketamine from various biological and beverage samples. These samples can be contained in the whole blood, dried blood stains and plasma; and the less expected ones such as breast milk; urine; saliva; hair and beverages such as orange juice; rose wine. Application of modern methods such as infrared (IR), nuclear magnetic resonance (NMR), ultraviolet-visible (UV-Vis) spectroscopy, electrochemical impedance spectroscopy (EIS) and mass spectrometry (MS) have shown quite efficiency in the detection of these compounds. These systems are further compounded with chemometric methods including PCA and PLS allowing them to solve different signals that overlap in a matrix of a complex mixture. This integration has not only increased accuracy but also made portable screening devices which could be used for real-time screening in both forensic and clinical environments.

The selection of an appropriate analytical method depends on many factors and it is not uniform. Factors including physico-chemical properties of the drug, time elapsed since the drug was taken, availability of the biological matrix and the expected concentration of the drug. Among the techniques available, LC-MS/MS has been widely recognised for investigating DFSA because of its superior sensitivity, versality and selectivity. Its high selectivity makes it valuable in particularly biological matrices which are complex in nature such as blood and urine where interferences can otherwise occur. Furthermore, GC-MS is often given preference in cases where volatile or semi volatile compounds are suspected due to their robustness and reproducibility. HRMS and TOF offer an additional advantage in untargeted screening. This should be done in cases where the specific drug is unknown. For hair samples, LC-MS/MS and HRMS are mainly well suited due to their high sensitivity and ability to handle complex matrices.

Furthermore, there are still several limitations that are present. The ePAD has low resistance to organic solvents, which

limits the analysis of samples in non-aqueous media. This could restrict the types of beverages or matrices that can be effectively analysed. Matrix effects from drink such as wine and juice also mask some of the drugs such as ketamine reducing both sensitivity and specificity. Identifying such drugs as GHB is very difficult since they have short biological half-lives and are present in very low concentrations of biological samples. Moreover, these analyses need advanced machinery and programs which are costly and may need a professional and specialised person, thus in many forensic laboratories these analyses may not be easily employed. However, there remain many challenges and these include the difficulties of translation of technology from one field to another and the issues dealing with increasing the sensitivity of instruments.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

DECLARATION OF AI-ASSISTED TECHNOLOGIES

During the preparation of this manuscript, the authors used an AI-assisted tool(s) to improve the language. The authors reviewed and edited the content and take full responsibility for the published work.

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