The Application of LC-MS in Forensic Toxicology

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Abstract: Analyses by LC-MS are of increasing importance in forensic toxicology and related fields. Some specific applications are discussed including the analysis of some drugs of abuse and other toxicologically highly relevant drugs like benzodiazepines, buprenorphine, LSD, muscle relaxants and opiate glucuronides.

Keywords: Forensic toxicology · LC-MS

Introduction

There are many substances which have insufficient volatility for direct identification by gas chromatography coupled to mass spectrometry, the well-established standard in forensic analysis. Such compounds need to be derivatized or hydrolyzed to obtain information on their identity. LC-MS is nowadays becoming an alternative method for the unambiguous identification of such substances [1–4]. Amongst them there are very important low-dosed drugs and metabolites such as the group of benzodiazepines or opiate glucuronides.

Ionization Modes

Two complementary interfaces for the transformation of the analytes from the liquid phase to the gas phase are available for most LC-MS apparatus: Electrospray ionization (ESI) interface and atmospheric-pressure chemical ionization (APCI) interface.

In an ESI interface, the column effluent is nebulized into an atmospheric-pressure ion source. Nebulization takes place due to the application of a high electric field and is helped by a strong stream of nitrogen gas. The solvent from the HPLC breaks into fine threads which subsequently disintegrate into small droplets. Analyte ions are generated in the ion source due to an ion evaporation process [5]. The analytes need to be ionizable in solution. Molecules of high polarity and molecular mass up to several thousand units such as peptides can be transferred by ESI.

In an APCI interface the effluent from the HPLC is nebulized into a heated vaporizer tube assisted by nitrogen gas where the solvent evaporation is completed. After entering the atmospheric pressure ion source, APCI is initiated by electrons generated at a corona discharge needle. The solvent vapor acts as a reagent gas. APCI is suitable for compounds of moderate polarity and molecular mass.

In contrast to GC-MS based on electron ionization (EI), LC-MS with an ESI or APCI interface is a very soft ionization technique comparable to chemical ionization in GC. Usually only ‘quasi-molecular’ ions are detected, where the molecules of interest are simply protonated or form adducts with other ions. As a consequence only little information on the structure and the identity is obtained. To get the required information collision-induced dissociation (CID) at atmospheric pressure in the ion source is possible in all equipment. Some also offer MS-MS as triple-stage quadrupole or MS² as ion trap detector.

In practice most analytes have to be tested to determine whether ESI or APCI generate more ions thus resulting in better sensitivity.

Library

A big advantage of GC-MS is the uniformity of the EI spectra collected at conventionally 70 eV, no matter where and with which equipment they were generated. For LC-MS the comparability of the obtained fragmentation spectra generated by CID or MS-MS remains an unresolved problem although some work is currently being undertaken to overcome this drawback [6–8]. These spectra depend very much on the geometry of the apparatus used. Also GC-MS-MS spectra almost always differ from LC-MS-MS spectra. The MS-MS spectra of midazolam, the active substance of the benzodiazepine drug Dormicum®, illustrates this phenomenon (Fig. 1): When a GC-MS-MS experiment with an ion trap (GCQ) is executed on the molecular ion (m/z = 325), the main fragment is formed by the loss of a methyl group (m/z = 310). Performing a LC-MS-MS experiment with an ion trap (LCQ Duo) on the protonated molecular ion (m/z = 326) yields a main fragment where chlorine has been eliminated (m/z = 291).

LC-MS vs. LC with Photo Diode Array (PDA) Detection

Although LC-MS has a much higher identification power there is a good reason to perform LC-PDA too. There are some compounds which give a much higher response with the PDA detector compared to the MS detector (Fig. 2). A typical example is caffeine. Although not very important in forensic toxicology, it
shows very well what can happen in a general unknown analysis. If only LC-MS is performed, caffeine might not be identified whereas its presence is easy to detect with PDA. Another compound showing the same effect but with toxicological importance is clobazam, the active ingredient of the drug Urbanyl®, a 1,5-benzodiazepine derivative used as sedative, anticonvulsant, and anxiolytic agent. The authors recently noticed that this drug and its metabolite are badly detectable with the routine LC-MS procedure in their laboratory, whereas analyzing the same blood extract with the LC-PDA procedure revealed no problem in the identification and quantification of clobazam.

Fig. 1. Comparison of GC-MS-MS with LC-MS-MS on the example of midazolam

Fig. 2. Chromatogram of an extracted blood sample containing 10 µg/l midazolam (m/z 326, rt 17.54) and metabolite (m/z 342, rt 15.27) as well as a non-quantified amount of caffeine (rt 6.03)
Determination of Drugs and their Metabolites in Biosamples by LC-MS

Sample preparation is required prior to any analysis. Mostly it consists of a sample extraction procedure on liquid–liquid (LLE) or solid phase (SPE) basis which cleans up and concentrates the compounds of interest. This extraction step is followed by a chromatographic step, in which the substances are separated from each other and from the remaining matrix interferences.

Benzodiazepines

Since the sixties benzodiazepines have been used as tranquillizers as a replacement for barbiturates. Nowadays they belong to the most frequently prescribed psychoactive drugs. The substance class counts more than 30 members, many of which form active and inactive metabolites. The benzodiazepines act as hypnotics, sedatives, and anxiolytics. Some of the most popular drugs of this class are diazepam (Valium®), flunitrazepam (Rohypnol®) and midazolam (Dormicum®). The range for the therapeutic dosage varies over 4 orders of magnitude from 0.5 µg/l for flunitrazepam to 3 mg/l for chlor Diazepoxide [9]. The metabolites of the low-dosed benzodiazepines in particular are detected preferably in MS-MS mode [10].

The authors’ procedure in case of suspected benzodiazepine intake is based upon LLE with butyl chloride followed by LC-PDA and LC-MS analysis. This serial detection assures that samples including substances with low ionization affinity may be detected and quantified by PDA whereas traces of drugs with good ionization affinity are identified by LC-MS. In several experiments the APCI ionization mode has been shown to be more suitable for this class of substances than the ESI mode.

Fig. 2 shows serial chromatograms of an extracted blood sample. By PDA detection the concentrations of the benzodiazepine midazolam and its metabolites are below the detection limit. But by applying the filters for the protonated molecular masses in the MS detection, low levels of midazolam and its metabolites can be detected.

A great feature is the possibility of a so-called ‘data-dependent scan’, meaning that as soon as an ion is detected above a defined threshold value the following scan will perform a MS-MS experiment on that ion. With this technique it is possible to get an overview of all extracted and detectable substances in the sample of question in full scan mode combined by an unambiguous identification in MS-MS mode (Fig. 3).

Buprenorphine

In France many addicts to heroin are treated with Subutex® , a sublingually administrated drug, with buprenorphine (B) as the active substance. This drug is metabolized in blood to nor-buprenorphine (NB) in which the parent drug has been demethylated.

The quantification is of great importance specially in cases concerning driving under the influence of drugs (DUID). The screening of urine samples is also performed with LC-MS because in the authors’ laboratory no immunoassay is available to test for buprenorphine. Several procedures have been published in recent years [11][12].

Blood levels are usually very low, such that no ‘data-dependent MS-MS experiment’ can be performed to make the required identification. As a consequence MS-MS experiments on the protonated molecular masses must be performed in the time windows of the elution of B and NB. Best results were obtained with SPE followed by LC-ESI-MS-MS. Deuterated isotopes of the drug and its metabolite are added to the samples as internal standards prior to the extraction for quantification (see Fig. 4). Therapeutic blood levels are between 0.5 µg/l and 5 µg/l. Blood levels measured in the authors’ laboratory range from 0.3 to 2.9 µg/l (B) and 0.1–9.8 µg/l (NB). Also urine levels may be low requiring the same treatment. Levels in urine were measured from 0.1 to 146 µg/l (B) and 34 to 657 µg/l (NB). After deglucuronidation of the urine samples much higher levels are observed.

LSD

The hallucinogenic drug LSD is extremely difficult to detect by GC because the active blood levels are very low and adsorption phenomena may play an important but negative role [13][14]. As a
consequence LC-MS-MS is the method of choice. Until now two metabolites are known to be formed by humans; 2-oxo-3-hydroxy-LSD and nor-LSD. Typically the drug contains also the diastereoisomer iso-LSD as an impurity, formed during the synthesis from lysergic acid. In humans this diastereoisomer is biotransformed to nor-iso-LSD. Sample work-up may consist of LLE in which the parent drug, the diastereomer and all metabolites are captured. Quantification of the impurities and metabolites with LC-ESI-MS is very difficult as only LSD itself is available in deuterated form.

As for other analytes in forensic analyses an immunoassay screening for LSD in urine is performed, followed by a chromatographic confirmation. But it is well known that some LSD immunoassays give a false positive result when e.g. chlorpromazine, the active substance of the neuroleptic drug Chlorazin®, or ambroxole, an expectorant substance of several drugs in the form of Muco- solvon®, is present. This can explain why in the authors’ laboratory most of the urine samples with positive LSD immunoassay result cannot be confirmed by LC-MS.

Many procedures have been published in recent years [15–17]. In the two years since confirmation analyses can be performed in the authors’ laboratory only seven samples proved positive. Table 1 summarizes the measured levels in blood and urine samples analyzed in the authors’ laboratory. In no case could 2-oxo-3-hydroxy LSD be identified in blood samples. Nor-LSD values range from not detectable to trace levels only. In urine samples the levels are much higher for all metabolites and also for the parent drug. Fig. 5 shows a chromatogram of an extracted urine sample containing LSD (0.25 µg/l), nor-LSD (1.3 µg/l), 2-oxo-3-hydroxy-LSD (30 µg/l), iso-LSD (13 µg/l), and nor-iso-LSD (2.1 µg/l).
gram of an extracted urine sample containing LSD with metabolites and impurities. The chromatographic separations of LSD and iso-LSD as well of nor-LSD and nor-iso-LSD are of importance because the diasteroisomers have identical fragmentation paths leading to fragments of equal m/z ratios. Monitoring typical fragments without chromatographic separation is thus inadequate.

Muscle Relaxants

Muscle relaxants are used in surgical medicine to help intubate patients and to keep them relaxed. In forensic toxicological analyses they are observed rather rarely, almost exclusively in combination with suicides. Chemically these drugs are salts of quaternary ammonium ions, some of which are doubly charged. LC-ESI-MS is the method of choice to analyze this class of substances which are already dissociated in aqueous solution. Sample preparation is based on SPE after addition of an internal standard, preferentially another muscle relaxant not present in the sample [18]. Typical examples of muscle relaxants are given in Table 2.

To adapt the LC method to the separation columns of the authors’ laboratory (Fig. 6) an inconveniently strong buffer (250 mM) for MS analysis is afforded, requiring extensive cleaning of the low pressure section of the apparatus. MS-MS is just one possibility for the identification (Fig. 7). The typical isotope ratio of these large molecules is a further possible identifier (Fig. 8).

Glucuronides of Opiates

The metabolism of heroin in blood leads via 6-acetylmorphine to morphine and subsequently to two glucuronides, morphine-3-glucuronide (M3G), an analytically inactive compound, and morphine-6-glucuronide (M6G) which has about the same analgesic power as morphine. Because the heroin on the illegal street market always contains other opium alkaloids like codeine, the method should be able to also identify this chemically related opiate and its main metabolite codein-6-glucuronide (C6G).

Heroin users develop a tolerance towards opiates. As a consequence expected blood levels may range from the limit of detection (10 µg/l) to about 2000 µg/l for the glucuronides, depending on the dose and time of the last intake. The early elution of M3G may cause problems during the analysis. It is important to separate it from the endogenous substances not eliminated during the sample preparation, otherwise ionization suppression is likely to occur leading to extreme loss in sensitivity. Deuterated standards of all compounds of interest are available making quantification results reliable. Fig. 9 shows the composed chromatogram of a blood sample taken from a deceased heroin abuser. For a better reading the traces for the internal standards are not shown. Several validated procedures dealing with quantifying the mentioned analytes have been published [19][20].

Summary

Many LC-MS procedures for the identification and quantification of drugs of abuse as well as toxicologically relevant drugs and their metabolites in bio samples are cited in the literature. Nevertheless LC-MS procedures cannot yet be used to perform general unknown screenings. The technique is complementary to GC-MS, but not yet really competitive. As long as the users are aware of the existing drawbacks, e.g. no universal library or ion suppression phenomena, LC-MS will help forensic toxicologists in their routine work.

Table 2. Frequently used muscle relaxants in Switzerland

<table>
<thead>
<tr>
<th>Brand name</th>
<th>Muscle relaxant</th>
<th>Charge in aqueous solution</th>
<th>m/z of main isotope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pavulon®</td>
<td>pancuronium</td>
<td>2+</td>
<td>286.2</td>
</tr>
<tr>
<td>Esmeron®</td>
<td>rocuronium</td>
<td>1+</td>
<td>529.5</td>
</tr>
<tr>
<td>Lythenon®</td>
<td>suxamethonium</td>
<td>2+</td>
<td>145.2</td>
</tr>
<tr>
<td>Tracrium®</td>
<td>atracurium</td>
<td>2+</td>
<td>464.3</td>
</tr>
<tr>
<td>Mivacron®</td>
<td>mivacurium</td>
<td>2+</td>
<td>514.4</td>
</tr>
</tbody>
</table>

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Fig. 7. Upper spectrum: MS-MS of rocuronium; lower spectrum: MS-MS of pancuronium.

Fig. 8. Mass spectra of pancuronium (mass of main isotope 572.5, m/z = 2) and rocuronium (mass of main isotope 529.4, m/z = 1) collected in zoom scan mode, providing high mass resolution.

Fig. 9. Composed chromatogram of an extracted post-mortem blood sample taken from a deceased heroin user: M3G (1200 µg/l), M6G (480 µg/l), morphine (180 µg/l), C6G (190 µg/l), codeine (30 µg/l) and 6-AM (10 µg/l).