A General Unknown Screening for Drugs and Toxic Compounds in Human Serum

Thesis

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1. LIST OF ABBREVIATIONS

Ac	alternating current
ACN	acetonitrile
amu	atomic mass unit
APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
AUC	area under the curve
CID	collision induced dissociation
c_{max}	peak concentration
CNLS	constant neutral-loss scanning
CV	coefficient of variation
DAD	diode array detection
Dc	direct current
DDA	data-dependent acquisition
DIS	daughter-ion scanning
EMIT	enzyme multiplied immunotechniques
ESI	electrospray ionization
FAB	fast atom bombardment
GC	gas chromatography
GUS	general unknown screening
HPLC	high performance liquid chromatography
IS	internal standard
LC	liquid chromatography
LLE	liquid-liquid extraction
LOD	limit of detection
ME	matrix effect
MRM	multiple-reaction monitoring
MS	mass spectrometry
PB	particle beam
RF	radio frequency
RI	retention index
RP	reversed-phase
RT	retention time

RRT	relative retention time
SIM	selected-ion monitoring mode
SPE	solid phase extraction
SRM	selected-reaction monitoring
STA	systematic toxicological analysis
$t^1/_2$	half-life
TIC	total ion current
t_{max}	time to peak concentration
TS	thermospray
TOF	time-of-flight
UV	ultra violet
XIC	extracted ion chromatogram

2. SUMMARY

Screening for a wide range of drugs and toxic compounds in biological samples is an important task for forensic and clinical toxicological laboratories. Our object was to develop a method to detect and identify a wide range of compounds by using HPLC-DAD and LC-MS.

A solid-phase extraction procedure using polymer-based columns was developed for the HPLC-DAD procedure. The extraction method appeared very universal. Ninteyfour of 100 drugs were extracted with a recovery of more than 50%.
For the LC-MS procedure, the Prospekt solid-phase extraction was chosen for its ability to be linked to the atmospheric pressure chemical ionisation (APCI) ion source. With this procedure all of the tested drugs with LC-MS were successfully extracted.

Hundred compounds from a variety of classes were investigated, if they can be identified and detected at low toxic serum concentration. Limits of detection were determined in spiked serum samples.

It was found that HPLC-DAD was able to detect 61 out of 100 compounds with our procedure at low toxic serum concentration. The limit of detection (LOD) for the majority of the tested drugs (76%) was ≤ 1’000 ng/mL. Drugs and toxic compounds were detected by comparison of the retention time and UV spectra with references compounds stored in a library.

The LC-MS instrument was operated in the positive and in the negative mode using data-dependent acquisition. Tandem mass spectrometry (MS-MS) was applied to identify toxicologically relevant substances in serum. An application program was created in order to detect automatically the unknown compounds. Drugs and metabolites were identified on the basis of their relative retention times, pseudo-molecular ions and fragment ions. A total of more than 400 spectra of more than 350 compounds were recorded. The corresponding relative retention times were added to the spectra in the constructed libraries (one for the positive and the other library for the negative mode).

Eightyseven drugs of compounds were identified from serum using on-line solid-phase extraction with LC-MS-MS. The limit of detection (LOD) for the majority of compounds (67%) was ≤ 100 ng/mL, ranging from 10 to 4000 ng/mL.

With the presented fully automated data-dependent LC-MS-MS procedure drugs can be analysed in serum with a high specificity and sensitivity. The LODs were sufficiently low to detect compounds at low toxic concentrations in serum. The integrated software drastically reduced the interpretation time. It was demonstrated that with the DDA-mediated-LC-MS-MS screening approach almost all of the drugs detected by the conventional techniques as well as additional drugs were identified. This technique is useful for GUS and confirmation analysis in clinical and forensic toxicology. In general, LOD for compounds are lower with the LC-MS procedure than the HPLC procedure. For compounds not able to detect with the LC-MS procedure such as analgesics and barbiturates HPLC-DAD appeared to be the complement method.

3. INTRODUCTION

3.1. GENERAL UNKNOWN SCREENING

The identification of unknown compounds taken during an acute poisoning episode is a challenging task in clinical and forensic toxicology. The screening for drugs and toxic compounds is called general unknown screening (GUS) or systematic toxicological analysis (STA). This procedure is an analytical method aimed to detect and identify unknown xenobiotics in biological fluids.

Acute poisoning with drugs is one of the most often occurring types of intoxication with exogenous compounds. In the year 2003, the number of intoxication incidents was about 24’000 in Switzerland. Drugs are the reason for two-thirds of the poisonings in Switzerland with an assured medical diagnosis. The poisonings can be voluntary (suicidal intention) in 22% or accidental (mostly children) in 72% of the cases according to the annual report of the Swiss Toxicological Centre.

Following the usual course of STA, samples will initially be analysed by immunochemical techniques (enzyme multiplied immunotechniques (EMIT), fluorescence polarization immunoassays, etc) (1, 2). These preliminary immunochemical screening procedures mainly concern rapid-response analytical tools providing a binary yes/no response, which indicates whether the target analytes are present above a preset concentration threshold or not. The samples providing a „yes“ response to one or more compound classes or target substances are analysed with a conformation method.

Apart from the first-line automated immunoassays available for the most common drugs, the GUS procedures currently used in clinical and forensic toxicology involve chromatographic techniques, ideally coupled to specific detectors (i.e., gas chromatography-mass spectrometry (GC-MS) (3) or high performance liquid chromatography (HPLC) coupled to diode array detection (DAD) (4-6)).

None of these methods is sufficient to identify all possible toxic compounds. GC is limited to apolar, volatile and thermally stable compounds (7). For instance, some polar compounds with no or little UV absorbency will neither be detected by GC-MS nor by HPLC-DAD. MS is more specific and reliable than DAD. The coupling of MS to HPLC seems to be a possibility to increase the range of compounds amenable to MS (8).

3.2. CHOICE OF SPECIMEN

The choice of specimen is often dictated by the clinical situation. The most common specimens used for the screening of drugs are blood, serum/plasma, and urine. Blood, plasma and serum are interchangeable in most methods. Urine is the most frequent specimen used in most hospital situations and may require hydrolysis prior to the isolation procedure to convert drug conjugates (glucuronides) to more easily measurable compounds. In forensic medicine the most investigated specimen is whole blood.

3.3. EXTRACTION TECHNIQUES

Chromatographic techniques require a preanalytical isolation procedure to separate drugs from a biological matrix. There are two main suitable procedures for a GUS: liquid-liquid extraction and solid-phase extraction. The extract can be concentrated and dissolved in an appropriate volume of mobile phase.

In some cases filtration or precipitation of proteins with an organic solvent (such as acetonitrile) prior to injection can provide a more direct means to introduce a sample into a HPLC. But these extraction procedures are not sufficient for a GUS.

3.3.1. Liquid-Liquid Extraction

This has been the traditional method for isolating drugs from biological specimen. A distribution of the solute occurs between two not mixable liquids (specimen as the aqueous phase and a solvent as the organic phase). The isolation has to be performed at a pH at which the analyte is uncharged. For acidic drugs the aqueous phase has to be acidified, for basic drugs the aqueous phase has to be basified. In liquid-liquid extraction (LLE) ethyl acetate, acetone, chloroform, toluene, dichloromethane, butyl acetate and diethyl ether have been used as organic phases (9). The extraction power of these solvents differ not much from each other (10).

Although LLE is still used today, there are a number of drawbacks that limit its usefulness. Liquid-liquid extractions are difficult to automate. So this extraction procedure is labour intensive and time consuming. It’s more difficult to reproduce the resulting data than with SPE (11). Emulsion formation, increased solvent use and subsequent waste leading to environmental and safety issues are additional problems of LLE. These drawbacks have led to the development of other separation methods such as solid-phase extraction (SPE).

3.3.2. Solid-Phase Extraction

SPE on columns consists of four basic steps. Condition to wet and activate the column packing bed is the first step. Then the analytes of interest are loaded on the column to allow retention. In SPE, the analytes to be extracted are partioned between a solid and a liquid. They must have greater affinity for the solid phase than for the sample matrix. Ideally interfering compounds are rinsed off during the washing step and then the analytes are desorbed with a solvent appropriate for instrumental analysis. The principles of separation involve intermolecular forces between the analyte and the sorbent, the liquid phase or sample matrix. These intermolecular forces are dipole-dipole forces, hydrogen bonding, ionic interactions and Van der Waal forces.

The main sorbents in GUS used for solid phase extraction are reversed-phase and ion-exchange.

Reversed-phase (RP) chromatography partitions organic solutes from a polar phase (generally aqueous) to a non-polar phase, which may be in form of a hydrocarbon chain or polymeric sorbent. It involves non-polar interaction of the solute with the stationary phase through low-energy Van der Waals forces. The affinity of the solute for the sorbent depends upon its hydrophobicity. The solute is eluted with organic solvent, often acetonitrile, ethyl acetate or methanol. The most common RP sorbents for SPE are chemically bonded silica phases (12-14). Most widely used bonded phases are C_{18} and C_8 sorbents (15). Pre-treatment is necessary with solvents such as acetonitrile or methanol.

Other sorbents used for RP extractions include highly cross-linked co-polymers (styrene-divinylbenzene) (13, 16). Compared with silica sorbents, these sorbents are generally more hydrophobic, more retentive, stable over a larger pH range and do not have secondary (unbounded hydroxy silanol) groups. They can be used in automated on-line combined extraction and analysis systems.

Oasis HLB (Waters) combines hydrophilic N-vinylpyrrolidone and lipophilic divinylbenzene, providing a hydrophilic-lipophilic balance. That means these cartridges have a large range to interact with different substances. This sorbent requires no conditioning step. Drying out does not destroy the structure of the sorbent compared to others (13).

In ion-exchange sorbents, compounds are extracted by a high-energy ionic interaction with the sorbent. Strong anion-exchangers retain anions by quarternary amines as exchange groups, cation-exchangers groups retain cations by sulfonic acids. For extraction, the pH of the samples is adjusted so that the solute molecules are ionized. They are absorbed at oppositely charged sites of the sorbent. Changing the pH elutes them. Mixed-mode sorbents have both non-polar and ion-exchange functional groups. The combination is chosen so that an analyte is retained by both mechanisms (15).

The SPE mechanism is more selective than liquid-liquid extraction because it is based on interaction between sample components and the sorbent, as well as on solubility. The more selective retention mechanism of SPE has a number of advantages over liquid-liquid extractions such as cleaner extracts, increased selectivity for the compounds of interest, no emulsion formation, larger selection of solvents available for use, and smaller volumes of solvent may be used for extraction. In general SPE can be used for smaller sample sizes. SPE also is less time consuming, and the procedure can be automated. Because of the short analysis time, volatile compounds also may be analyzed without significant loss (17).

The majority of methods have used silica-based columns. Although successful, silica-based sorbents may have a number of drawbacks. First, for bonded phases, the reaction between the organosilane and the silica is incomplete, leaving unreacted silanol groups that can act as cation-exchange sites and creating a secondary retention mechanism. End-capping reduces the amount of free silanols by converting the hydroxyl group to a methoxy group but it is not 100% effective. Second, the sorbent is not stable at pH extremes. At pH below 2, there can be breakdown of the silyl ether linkage.

The polymer-based sorbents were developed to overcome the limitations of silica-based sorbents. The water-wettable sorbent is promoted for its ability to retain a wide spectrum of polar and nonpolar compounds and to remain stable from pH 1 to 14 (13).

The automation of solid-phase extraction has different benefits. Precision, recovery and accuracy with fewer operator errors, reduced analysis time are some of these advantages (12, 14). In addition the automated SPE system can be connected directly to an analyser. Disadvantages can be the risk of carry-over and the loss of sensitivity (18).

3.4. CHROMATOGRAPHY

An autosampler is used to inject samples automatically into the liquid chromatography (LC) system. The high performance liquid chromatograph separates a sample into its chemical components by liquid chromatography. In liquid chromatography, the sample mixture partions between a solid stationary phase (column) of large surface area and a liquid mobile phase that passes through the column. The chemical properties of the components, the column and the mobile phase determine when each component elutes from the LC and enters the DAD system or the MS.

3.4.1. HPLC-DAD System

Diode-array ultraviolet detectors record the absorbance of compounds over a range of wavelengths (e.g., 200-400 nm) as they pass through the detector flow cell, thus allowing the on-line acquisition of UV spectra.

The HPLC-DAD system has been extensively shown to be an efficient and practical method for both clinical and forensic toxicological investigations. This system is able to screen for a wide range of compounds including benzodiazepines, analgesics, diuretics, tricyclic antidepressants, drugs of abuse (amphetamines, cocaine) and barbiturates. Elliott and Hale had shown a screening of basic, acidic, and neutral drugs, providing reproducible results (19).

Using the diode-array facility, it is also possible to detect any probable metabolites, which are not available as drug standards by comparing UV spectrum of the proposed metabolite and the parent compound. Some phase-one metabolic transformations (especially N-dealkylation and sometimes hydroxylation) do not alter the UV chromophore of a drug. The system and the retention index (RI) database should be combined with additional techniques such as liquid chromatography-mass spectrometry, thus providing a powerful identification tool that combines RI values, UV spectra, molecular masses and ion fragmentation spectra.

3.4.2. LC-MS System

The LC-MS system consists of an atmospheric pressure ionization (API) source, ion optics, mass analyzer, and ion detection system. Ionization of the sample takes place in the API source. The ions produced in the API source are transmitted by the ion optics into the mass analyser. Selected ions are ejected from the mass analyzer and reach the ion detection system where they produce a signal. Fig. 1 shows a schematic drawing of a liquid-chromatography mass spectrometer system.

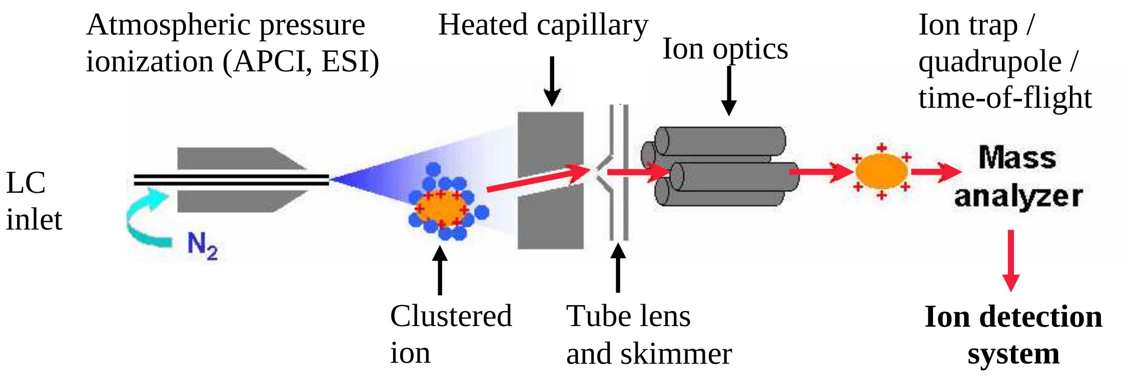


Fig. 1. Schematic drawing of a liquid-chromatography mass spectrometer.

3.4.2.1. Interfaces

LC-MS interfaces remove the mobile phase and ionize the analyte. Different LC-MS interface types such as moving belt, direct liquid introduction, continuous-flow or frit-terminated fast atom bombardment (FAB), particle beam (PB), thermospray (TS), electrospray (ESI) and atmospheric pressure chemical ionization (APCI) were developed. PB, FAB or TS have several limitations such as less sensitivity or less universality. Today, two relatively robust LC-MS interfaces types seem to have become the golden standards of LC-MS, the atmospheric pressure ionization techniques, ESI and APCI.

ESI and APCI have been used for a large majority of the applications of LC-MS in the last years. ESI and APCI involve a soft ionization process, which can be overcome by using collision-induced dissociation, which provides thorough fragmentation of the compounds.

3.4.2.2. Electrospray Ionization

The ESI mode transfers ions in solution into the gas phase. Many samples, which were previously not suitable for mass analysis (for example, heat-labile compounds or high molecular weight compounds) can be analyzed by ESI. ESI typically produces mass spectra consisting of multiply charged ions depending on the structure of the analyte and the solvent.

ESI can be used to analyze any polar compound that generates an ion in solution. Generated ions include adduct ions (for example, polyethylene glycols can be analyzed from a solution containing ammonium acetate, because of the adduct formation between the NH_4^+ ions in solution and oxygen atoms in the polymer). With ESI the range of molecular weights that can be analyzed is greater than 100'000 atomic mass units (amu), due to multiple charging. So proteins as well as peptides can be analyzed.

The flow rate from the LC into the MS detector can vary over a range of 1 µL/min to 1000 µL/min. Transported by the mobile phase the sample enters the ESI needle. To the needle a high voltage (+/- 5 kV) is applied. The needle with the aid of nitrogen gas sprays the sample solution into fine mist droplets. These droplets are electrically charged at their surface. Solvent evaporates from the droplets. The electrical charge density increases up to a critical point known as the Raylight stability limit. At this point, the droplets divide into smaller droplets (20, 21). This is because the electrostatic repulsion is greater than the surface tension. The process is repeated many times. Finally, sample ions are ejected from the cluster ions into the gas phase. This takes place in a heated capillary.

The ESI process is affected by droplet size, surface charge, liquid surface tension, solvent volatility, and ion solvation strength. Large droplets with high surface tension, low volatility, strong ion solvation, low surface charge, and high conductivity prevent a good electrospray process (20).

3.4.2.3. Atmospheric Pressure Chemical Ionization

Like ESI, APCI is a soft ionization technique. APCI provides molecular weight information for compounds of medium polarity that has some volatility. APCI is typically used to analyze molecules with molecular weight up to 2’000 amu.

APCI is a gas phase ionization technique. Therefore, the pH of the analyte in the gas phase and solvent vapour play an important role in the APCI process. APCI is a very robust ionization technique. It is less affected by minor changes in most variables such as changes in buffer and buffer strength. The rate of solvent flowing from LC into the MS detector in APCI mode is typically high (between 0.2 and 2 mL/min) (22).

The APCI nozzle vaporizes the sample solution in a high temperature tube. The needle is located near the end of the tube. A high voltage is applied to the corona discharge needle. Reagent ions are formed through a series of chemical reactions. First primary ions are formed. These primary ions can be nitrogen, oxygen gas or solvent molecules. The primary ions react with the molecules in the sample to form ions (21).

APCI as well as ESI can be used in positive or negative ion polarity mode. A positive applied voltage is used to generate positive ions and a negative applied voltage to generate negatives. For most molecules, the ion-positive mode produces a stronger ion current. This is especially true for molecules with one or more basic nitrogen (or other basic) atoms. Molecules, which generally produce strong negative ions, with acidic sites such as carboxylic acids and acidic alcohols, are an exception to this general rule. Although, in general, fewer negative ions are produced than positive ions, negative ion polarity can be more specific. This is because the negative ion polarity mode sometimes generates less chemical noise than does the positive mode. Thus, selectivity might be better in the negative ion mode than in the positive ion mode.

3.4.2.4. Ion Transfer Capillary and Ion Optics

The ion transfer capillary assists in desolvating ions that are produced by ESI and APCI probes. Ions are focussed into the ion transfer capillary in the atmospheric pressure region and transported to the skimmer region by decreasing pressure gradient and electrostatic forces. The ion transfer capillary can be heated. Typical temperatures are 150 to 200° C. Ions from the ion transfer capillary enter the tube lens. The tube lens has a dependent potential to focus the ions towards the skimmer. If the tube lens offset voltage is high, collisions with the background gas can be energetic enough to cause fragmentation. This fragmentation is called the ion source collision induced dissociation. The skimmer acts to reduce the number of neutral molecules and large charged particles. These particles would create detector noise.

Ion optics (in our apparatus octapoles) transmits ions from the API source to the mass analyzer. Magnetic fields are used to direct and focus the ion stream coming from the source.

3.4.2.5. Analysators and LC-MS Detection Modes

Mass analysis of ionized substances is performed using one or two mass analyzers (MS-MS), which consist predominantly of ion traps and quadrupoles, sometimes of sector field and time-of-flight instruments (TOF).

They can operate in the full scan mode or in the more sensitive selected-ion monitoring mode (SIM) detecting positive or negative ions. The MS-MS combination provides additional possibilities. Both analysers can be operated in a scan or in a selected-ion monitoring mode.

The four combinations are: Parent-ion scanning (scan mode in the first, SIM in the second analyzer), daughter-ion scanning (DIS; SIM in the first, scan mode in the second analyzer), constant neutral-loss scanning (CNLS; scan mode in both analyzers) or selected-reaction monitoring (SRM; SIM in both analyzers). DIS is preferable for the identification of drugs and or their metabolites in complex matrices. Separation is performed on the LC and in the first mass analyzer, while structural information is obtained by fragmentation in the second analyzer. SRM (also named multiple-reaction monitoring MRM) is the most powerful technique for quantification of small amounts of analyte in complex matrices (23, 24).

Quadrupole is a quadrilateral array of square rods that acts as an ion transmission device. A radio-frequency voltage and direct current (dc) offset voltage, that are applied to the rods give rise to an electric field that guides the desired ions along the axis of the quadrupole. The other ions collides with the four square rods. During ion transmission, the offset voltage is negative for positive ions and positive for negative ions. Quadrupoles can also be placed in tandem to perform fragmentaion. Fragmentation takes place between the two quadrupoles in the collision cell. The most common set-up is the triple quadrupole mass spectrometer.

The ion trap includes three electrodes: the entrance endcap electrode, the exit endcap electrode and the ring electrode. Both endcap electrodes have a small hole in their centre to permit the passage of ions into and out of the mass analyzer cavity. Various alternating current (ac) voltages are applied to the ring and endcap electrodes to trap, fragment, and eject ions according to their mass-to-charge ratios.

The processes that occur in the mass analyzer can be divided in four steps: ion storage, ion isolation (SIM, SRM, and MS/MS full scan), collision induced dissociation (SRM and MS/MS full scan), and ion scan out.

The application of a radio-frequency voltage to the ring electrode produces a three-dimensional quadrupole field within the mass analyzer cavity. This time-varying field drives ionic motion in both the axial (towards the endcaps) and radial (from the ring electrode toward the centre) directions. Ionic motion must be stable in both the axial and radial directions for an ion to remain trapped. During ion scan out, the system produces a mass-dependent instability to eject ions from the mass analyzer in the axial direction. The voltage at which an ion is ejected from the mass analyzer is called its resonance voltage.

Ac voltages that are applied to the endcap electrodes stimulate motion of the ions in the axial direction. The voltages applied to the endcap electrodes are equal in amplitude but are 180° out of phase to one another. When the radio-frequency applied to the endcaps equals the resonance frequency of a trapped ion, the ion gains kinetic energy and the ion is ejected.

During the collision induced dissociation a voltage is applied to the endcap electrodes to fragment ions into product ions. This voltage is not strong enough to eject an ion from the mass analyzer. However, ion motion in the axial direction is enhanced and the ion gains kinetic energy. The mass analyzer cavity contains helium as a collision activation partner. After many collisions with helium gas, the ion gains enough internal energy to cause it to dissociate into product ions.

3.4.2.6. Data-Dependent Acquisition (DDA)

In the first step (so-called "survey scan") the MS-MS instrument is operated in the full-scan single-mass mode using the second mass filter in order to select ions above a predefined intensity threshold. Instantly, the first mass filter is set to selectively transmit these high-intensity ions to the collision cell where fragmentation energy is switched on and the resulting fragments are analysed by the second mass filter in the scan mode. Finally after a short time the instrument switches back to the initial conditions, with the possibility to set a refractory period for the last ions selected. The major advantage of this approach is its high specificity and selectivity, as the spectra recorded come from a single parent ion.

It can be difficult to detect toxic compounds among background noise. The setting of a given threshold can be important due to the intense and highly variable background noise produced by extracts of real samples. If the threshold is given a high value it results in very poor sensitivity but high specificity, and if it is given a very low value it may result in too much "noisy" information.

3.4.2.7. Ion Detection System

The ion detection system includes a 15 kV conversion dynode and a channel electron multiplier. A potential of + 15 kV for negative ion detection or – 15 kV for positive ion detection is applied to the conversion dynode. When an ion strikes the surface of the conversion dynode, one or more secondary particles are produced. These secondary particles can include positive, negative ions, electrons, and neutrals. When positive ions strike a negatively charged conversion dynode, the secondary particles of interest are negative ions and electrons. When negative ions strike a positively charged conversion dynode, the secondary particles of interest are positive ions. These secondary particles are focused by the curved surface of the conversion dynode and are accelerated by a voltage gradient into the electron multiplier. Secondary particles from the conversion dynode strike the inner walls of the electron multiplier with sufficient energy to eject electrons. The ejected electrons are accelerated farther into the cathode, drawn by the increasingly positive gradient. Due to the funnel shape electrons do not travel far. They strike again the surface, thereby causing the emisson of more electrons. Thus, a cascade of electrons is created that finally results in a measurable current. The current is proportional to the number of secondary particles striking the cathode.

3.4.2.8. Vacuum System

Vacuum is necessary to perform and maintain a mass spectra analysis. At normal pressure the produced ions would collide with molecules (N_2, O_2, etc.) passing from the API stack to the ion detection system. High vacuum levels cause reduced sensitivity, and reduced lifetime of the electron multiplier. The vacuum system evacuates the region around the API stack, ion optics, mass analyzer, and ion detection system. A forepump establishes the vacuum necessary for the proper operation of the turbomolecular pump. The pump maintains a pressure of approximatively 1.33 mbar. A turbomolecular pump provides the vacuum for the ion optics and analyzer regions. Under normal operating conditions the vacuum in the API region is 1020 mbar and 2.67×10^{-5} mbar in the analyzer region.

3.4.2.9. Adduct Formation

Besides matrix effects, adduct formation also adds to the complexity of LC-MS. Generally, ESI or APCI result in deprotonated $(M-H)^-$ molecules in the negative mode and in protonated $(M+H)^+$ molecules in the positive ionization mode. Several adduct ions such as $(M+Na)^+$, $(M+K)^+$, or $(M+NH_4)^+$ were also reported (25, 26). The exact mechanism in adduct formation is not clearly understood, carboxyl or carbonyl ether or ester groups are believed to be responsible for binding the alkali metal ions. Adduct formation process is not reproducible and consequently it is not clear, what adduct ion can be used for MRM. Addition of ammonium results in $(M+NH_4)^+$. The first step in fragmentation is the loss of neutral NH_3. The latter can then fragment further. Sodium adduct ions are much more stable and yield less fragments. It is clear that the evaluation of both matrix effects as well as adduct formation should be included in the validation procedure of a LC-MS method.

3.4.3. Retention Time

It is unusual for a substance to have an absolute retention time that remains constant either over a period of time (because of a gradual change of stationary phase) or between different batches of columns with nominally identical packing material. In order to account for this variability, relative retention time or retention index (RI) can be used as a method of correction. They are based on the relationship between a substance's retention time and those of a reference compound. The use of retention index was shown to be advantageous (27).

3.4.4. Chromatographic Conditions

Drug screening is a qualitative technique for the identification of the presence of drugs. Gradient chromatography is needed for the analysis of the large diversity of substances. For quantitative LC analyses of compounds identified by screening, it can be more efficient to use isocratic chromatography.

3.4.5. Influences of Biological Matrix

3.4.5.1. Influences of Biological Matrix on Chromatographic Behaviour

Bogusz et al. showed that the co-extracted biological matrix did not exert any specific influence on the chromatographic behaviour (28). The biological matrix did not affect the chromatography of acidic, neutral and basic drugs analyzed by means of gradient HPLC. To identify drugs extracted from biological samples, it is possible to use the retention time of pure drugs.

3.4.5.2. Influences of Biological Matrix on Detection in HPLC

The presence of extracted matrix substances can affect the identification of drugs by HPLC with a diode array detector spectrum library. These compounds can co-elute at the same time as some toxicologically relevant substances and overlap the corresponding UV spectra. The purity of the biological extract is therefore of critical importance for successful HPLC-DAD identification.

3.4.5.3. Influences of Biological Matrix on Detection in MS

Matrix effect (ME) in mass spectrometry can be defined as any change in the ionization process of an analyte due to a co-eluting compound (29). This can result either in an enhancement or in a suppression of the ionization. Ion suppression or enhancement affects the precision, sensitivity and accuracy of an analytical procedure (30).

Sample matrix, co-eluting compounds, and cross-talk can contribute to ion suppression. ESI is more impacted to ion suppression than APCI (31).

In electrospray ionization (ESI) the ionization process is taking place in the liquid phase. Matrix effects in ESI are due to a competition of matrix compounds and molecules of interest for access to the droplet surface and subsequent gas-phase emission. Besides that, matrix constituents can change properties of the surface tension and viscosity. These factors are known to affect the ionization process (29).

In APCI, the ionization is taking place in the gas phase. The non-volatile matrix compounds are thought to co-precipate with the analyte molecule. This influences the ionization process (32).

In summary, ion suppression results form the presence of less volatile compounds that can change the efficiency of droplet formation or droplet evaporation, which in turn affects the amount of charged ion in the gas phase that reaches the detector.

3.4.5.4. Evaluation of Matrix Effects in MS

Matuszewski et al. described a procedure for the evaluation of matrix effects (33). Three sets of samples are necessary. Set A consists of neat standard solutions. For Set B blank matrices are supplemented (after extraction) with the same amount of standards as used for set A. Set C consists of extracts of different blank matrices, supplemented with the same amount of standards but added before extraction. Matrix effects (ME%), recovery (RE%) and process efficiency (PE%) can be calculated with different formula. For the calculation the resulting peak areas are needed.

$$ME\% = B/A \times 100$$

$$RE\% = C/B \times 100$$

$$PE\% = C/A \times 100$$

Bonfiglio et al. described another procedure to evaluate matrix effects (34). This procedure is based on the postcolumn infusion of an analyte in a chromatographic run of an extract or a blank matrix. This signal is compared to the signal obtained with the post-column infusion of this same model analyte in a chromatographic run with eluent only. This procedure indicates also critical areas in the chromatogram. Typical examples of substances known to influence the ionization process include salts and other endogenous compounds (fatty acids, triglycerides), dosing vehicles (polyethylenglycol, propylenglycol and cremophore), anticoagulants, and constituents of sampling material (e.g. polymers) (35, 36).

It was also demonstrated, that the ionization efficiency of a co-eluting internal standard (IS) is influenced by high levels of the compound of interest.

Sample preparation also influences the amount of ion suppression. Protein precipitation alone results in pronounced matrix effects over the whole chromatographic run. SPE was able to remove efficiently hydrophilic interfering compounds. But hydrophobic interferences were increased.

There are a few strategies to eliminate matrix effects, but often it is very difficult. A reduction of matrix constituents injected can help. This can be done by injecting a smaller sample volume, a diluted sample or by applying more selective extraction techniques. Then, co-elution of the analyte and matrix constituents should be avoided. But the separation on the liquid chromatographic system is limited.

The use of co-eluting internal standard(s) such as labelled ISs seems ideal because it is expected that the matrix effect on the analyte and on the IS is identical. But labelled internal standards are not always available and costs are often very expensive. In addition, these standards cannot be used in a GUS.

The mobile phase influences the ionization efficiency in LC-MS. Additives can also have effects on matrix induced ion suppression or enhancement of an analyte. The addition of small amounts of ammonium formate resulted in better ME% values. Higher levels or to high levels of other acids suppressed the signal (37).

3.5. MASS SPECTRA LIBRARIES

Gas chromatography-mass spectrometry was applied for GUS over the last decades. It was shown that GC-MS is very specific, sensitive and is able to build very large libraries of standardized spectra (3). A successful GC-MS procedure requires a volatile, thermally stable analyte. But GC-MS fails often to detect polar and thermally labile and non-volatile compounds. These requirements generally necessitate extraction from the biological matrix, followed in many cases by derivatization. These limitations led to investigate other possibilities for analyzing biological specimens (HPLC-DAD, LC-MS (-MS)).

3.5.1. Single Mass Spectrometry

A proposed alternative for a GUS is single MS spectra. The mass spectrometer works in the scan mode and applies in-source CID. The sample is screened at variable orifice voltages. The peaks exceeding a preset intensity are identified by comparison of their spectra with in-source CID spectrum libraries. The spectra obtained using in source-CID are mostly identically as those produced by conventional CID in the collision cell of a triple quadrupole mass spectrometry or of an ion trap.

Marquet et al. described a procedure using full-scan from 100 to 1’100 amu, with a step of 0.2 amu (38). In source CID was set at four continuously alternated voltages. In the positive mode CID was with low energy 20 eV, in high CID with high energy 80 eV, in the negative mode with low –20 eV, with high CID energy –80 eV. Several teams showed that fragmentation ensured reproducible in source CID spectra, at least using the same type of instruments (39).

To obtain informative spectra (fragment ions and protonated molecule respectively molecular ion) pairs of full mass spectra were reconstructed by adding spectra at 20 eV and 80 eV on one hand and –20 eV and – 80 eV on the other. These spectra were compared with spectra in the library. One library was built in the positive mode containing 1’100 reconstructed mass spectra; the other library was built in the negative mode containing 500 reconstructed spectra. Software was developed in co-operation with the manufacturer to automatically reconstruct such spectra and compare each positive and negative spectrum, together with their relative retention time to those in the library.

This LC-ES-MS technique was compared to GC-MS and HPLC-DAD GUS procedures for the identification of unknown compounds. 75% of the spiked compounds in these samples were identified by this method versus 66% for GC-MS and 71% for HPLC-DAD. The conclusion was that it was complementary to GC-MS and HPLC-DAD and helped enlarge the range of drugs detected (40).

Gergov et al. also built such a library of reconstructed CID-MS spectra using a triple-quadrupole mass spectrometry. They obtained the reconstructed spectra by the addition of spectra recorded at 25 eV and 90 eV (39). The authors concluded that the later mentioned MS/MS library showed better results. MS/MS spectra showed no interference caused by either co-elution of β-blocking drugs or matrix material as a result of its superior selectivity compared with single MS-CID. Better match indices were obtained.

Weinmann et al. built a spectra library of 400 drugs using a triple quadrupole mass spectrometry (41). In source CID was applied at 20, 50, and 80 V only in the positive mode. No reconstructed spectra were obtained. Each spectrum obtained was compared to the library. This procedure cannot be called GUS because selective extraction procedures were applied and when interfering compounds or background noise occurred, they used MS/MS fragmentation.

In summary, in-source CID mass spectra showed that they were reproducible with the same type of instruments (42). The main drawback of these techniques is the low specificity and selectivity, as the spectra recorded do not result from a single parent ion. To obtain good mass spectra of signals against a high background is almost impossible. This technique requires a completely separation of drugs and matrix compounds, which is not achievable.

Reports give hints of poor interinstrument reproducibility, which would require the development of mass spectral libraries for each brand. A standardization of the crucial parts of the instruments with respect to in-source CID would be necessary. This is one explanation of the absence of any large, commercial in-source CID mass spectral library so far (43).

3.5.2. Tandem Mass Spectrometry

Weinmann et al. built an MS-MS library of more than 500 therapeutic or illicit drugs using LC-ES-triple-quadrupole spectrometry (41). One or a few parent ions were selected in the first quadrupole (generally the protonated molecules in the positive ionization mode or the molecular ions in the negative mode). These ions were fragmented in the collision cell. The fragments were analyzed in the third quadrupole in the scan mode. Four different collision energies (20, 30, 40, 50 eV) were applied resulting in four different positive product-ion spectra. These spectra were recorded in the library for each compound.

Gergov et al. developed a library of MS-MS spectra for almost 400 therapeutic or illicit drugs (39). Generally collision energy of 35 eV was applied to obtain MS-MS spectra. Additional spectra were acquired at 20 or 50 eV for those compounds giving no informative spectrum at 35 eV. In the first step of the operation, samples were pre-screened using LC-MS-SIM. Any matches to a previously defined list of retention times and $[M+H]^+$ were recorded. From this match a multi-period LC-MS-MS-CID product ion method was automatically created, that means these ions were selected as parent ions for product-ion scanning and library searching. This is a rather complicated process for a β-blocking screening, when direct product ion-scanning could have been applied. This method described a preliminary version of a data-dependent acquisition.

The same authors found a high reproducability of MS-MS CID-spectra between the instruments (44). They were using three different triple quadrupole instruments from the same manufacturer. Their results suggested good interinstrument fragmentation reproducability.

Baumann et al built a MS-MS library of more than 500 spectra using an ion-trap mass spectrometer in the positive mode (45). To obtain rich product ion spectra, resonance excitation at 20 amu below the parent ion selected was used, to further dissociate the $[M+H-H_2O]^+$ ions. A mass dependent correction was automatically applied to the collision energy, because generally fragmentation energy decreases linearly when mass increases. Specific MS-MS spectra of different substances could be recorded.

The described libraries and methods are theoretically interesting but of little use in clinical toxicology. These procedures cannot be used as GUS methods, because samples are monitored only for a previously selected limited number of compounds. A solution to this limitation could be data-dependent acquisition.

3.5.3. Data Dependent Acquisition

The data dependent acquisition method was first used in a preliminary GUS procedure by Decaestecker et al (46). They used a time-of-flight detector in the positive electrospray mode. The quadrupole initially transmitted all masses (50 to 450). If an ion reached a predefined threshold the quadrupole selectively transmitted these high-intensity ions to the collision cell operating with single fragmentation energy. The resulting fragments were analyzed in the TOF detector. The instrument switched back to the initial conditions. A refractory period was applied to the last selected ions for 2 minutes.

Fitzgerald et al used also the technique of data-dependent acquisition (47). They modified the commercially available column-switching instrument, the REMEDi HS from BioRad Diagnostics, to make it compatible with atmospheric pressure ionization. Urine samples were injected directly on the column switching system. The compounds were analyzed in the full scan mode between 50 and 500 amu. When any ion exceeded a preset threshold, they were selected and fragmented by collision-induced dissociation in the ion trap.

The major advantage of these techniques is their high specificity and selectivity, as the spectra recorded come from a single parent ion. The main limitations of these works are that they do not explain the procedure to detect unknown substances. Also they did not mention the LOD of any substances.

In a further paper Decaestecker specified the different LOD of some drugs but still did not explain the mechanism of drug finding. They described that setting a low switching intensity threshold will increase the number of interferences detected. As a consequence, the interpretation of the data set was much more complicated. And no automated data interpretation was described.

Marquet et al described a preliminary procedure for the screening of drugs using a quadrupole-linear ion-trap mass spectrometer (48). For the first detection step (so called „survey scan“) an „enhanced“ MS mode was used. Ions were accumulated and then filtered in the Q3-linear ion-trap. MS-MS mode for on-the-fly fragmentation of the ions above the user-defined data-dependent acquisition was applied. Four in-source collision-induced fragmentation conditions are altered, low and high fragmentation in the positive and negative modes (20, 50, -15 and –40 V, respectively). Positive and negative reconstructed spectra were obtained by adding two spectra on the other. Better signal-to noise ratio was obtained with this method compared to the in source CID-MS technique of the same group; because the reconstructed mass spectra decreased the influence of contaminant ions.

3.5.4. Sample Preparation in a General Unknown Screening

Sample preparation is a key step in a screening procedure because the analytical system can not detect a compound if it is not extracted and so not injected in the system. The ideal extraction procedure would isolate the compounds of interest and eliminate the biological matrix compounds. The critical point is the signal-to-background noise ratio. The signal-to-background noise is determined by the height of the chromatographic peaks and the baseline (purity of the extracts).

Decaestecker et al tested an entire series of SPE sorbents for a LC-MS-MS general unknown screening (49). The sorbents were divided in three categories: apolar, mixed-mode and polymeric. The extraction procedure was formulated individual for each column. Only neutral and basic compounds were chosen.

From the apolar sorbents (Isolute C_2, C_4, C_8, C_{18}, C_{18} MF, PH and CN) the C_8 packing material demonstrated the best overall extraction yield. MCX gave the best extraction yields from the type from the mixed-mode category (MCX, HCX, HCX3, HCX5) and was slightly better than HLB from the polymeric category.

Maurer et al proposed two extraction procedures for a GUS one with LLE and the other with SPE (50-52). In the LLE procedure method the sample was extracted at pH 7 and at pH 12 with a mixture of diethyl ether/ethyl acetate (1:1, v/v). The organic phases were evaporated to dryness. The combined residues were dissolved in organic solution.

In the SPE method samples were worked up with HCX columns. The compounds of interest were eluted with methanol/aqueous ammonia (98:2, v/v). A drawback of this extraction procedure is that the methods are not suitable for all drugs. For example benzodiazepines could not be extracted in a sufficient way with the SPE procedure.

Venisse et al applied different extraction techniques using Extrelut, Oasis HLB and MCX cartridges. Both types of Oasis columns resulted in higher background noise, but the extraction and detection of tested compounds was much better than with Extrelut columns (53).

In summary, only liquid-liquid extraction and solid phase extraction method were used. Liquid-liquid extraction requires two procedures, one for acidic and one for basic drugs. SPE was performed with mixed-mode cartridges, classic hydrophobic bonded phase or a polymer.

3.5.5. Chromatic Conditions in a General Unknown Screening

Most commonly used stationary phases were reversed-phase C_{18} columns. A short guard column with the same type of stationary phase is recommended. In LC-MS the mobile phases consisted of mixtures of volatile buffers (e.g. ammonium acetate or formate) with acidic pH and organic modifiers such as methanol and acetonitrile. In HPLC-DAD the mobile phase consisted of phosphate buffer with acidic pH and acetonitrile or methanol. Gradient elution was performed.

3.6. RESEARCH OBJECTIVE

Acute poisonings account for 10 to 30% of the admissions to non-specialized care units, and 7 to 15% of autopsies performed in forensic institutions (4). Often, an intoxicated patient entering the hospital is consciousness or the patient or his relatives give misleading information. Consequently, the development of a reliable technique for general-unknown screening of pharmaceutical, toxicants and drugs of abuse in biological fluids is necessary. Such screening methods should be simple, rapid, reproducible, and available to cover a broad spectrum of toxicologically relevant substances, in one analytical run, with sufficient specifity. The combinations of mass spectrometry with suitable chromatographic procedures are the methods of choice, because they are very sensitive, precise, specific, universal and if coupled to automated extraction system very fast.

It is necessary to speed up the process of a STA. The results should be given automatically. The aim of the project was to develop a fully-automated LC-MS method in combination with a HPLC-DAD method to detect a wide range of toxicologically relevant compounds.

4. MATERIALS AND METHODS

4.1. GENERAL UNKNOWN SCREENING WITH HPLC

4.1.1. Materials

Test substances obtained from various pharmaceutical companies were of pharmaceutical purity. Organic solvents and reagents were of analytical grade. Acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Solid-phase extractions (SPE) were carried out on Oasis HLB cartridges (Waters, Rupperswil, Switzerland). Deionised water was generated with a Milli-Q water purification system from Millipore (Kloten, Switzerland).

4.1.2. Sample and Buffer Preparation Procedures

Separate stock solutions were prepared in methanol-water (1:1, v/v) at a concentration of 100 µg/mL. Standards in drug free serum (Biorad, Reinach, Switzerland) were prepared by spiking stock solutions of drug mixtures to make concentrations ranging from 0.250 to 5 µg/mL, resulting in a set of standards with the following concentrations: 0.250, 0.500, 1.000, 2.000 and 5.000 µg/mL.

The buffer (pH 2.2) was prepared with a 20 mmol/L KH_2PO_4 solution, adjusted to the desired pH by appropriate addition of orthophosphoric acid.

4.1.3. Extraction Procedure

One ml of serum was acidified by addition of 20 µL phosphoric acid 85%. The solid-phase extraction (SPE) procedure was carried out on Oasis HLB cartridges. The Oasis HLB cartridges were conditioned initially with methanol 1 mL, followed by 1 mL Millipore water. 1 mL per serum sample was loaded onto the cartridges. Cartridges were subsequently washed with 1 mL Millipore water and dripped dry under charging pressure. The compounds of interest were eluted with 1 mL methanol. The extracts were evaporated to dryness under a stream of nitrogen and then reconstituted in 1 mL of a 10% acetonitrile solution for the general unknown screening. For the quantification methods, the extract was solved in the corresponding mobile phase.

4.1.4. HPLC-DAD Method

A Varian Pro Star HPLC system was used for this analysis. It consisted of a Pro Star 230 solvent delivery module (SDM), a Pro Star 330 PDA Detector, and a Star chromatography workstation system with software version 5.50. The analytical column used was a LiChrospher 60 RP-select B (5 µm, 125 x 4 mm) protected by a LiChrospher 60 RP-select B (5 µm, 4 x 4 mm) both obtained from Merck (Darmstadt, Germany).

A HPLC method for the GUS and methods for the quantification of specific substance groups were developed. The gradient and the running time was method dependent. The HPLC was performed with a gradient of acetonitrile in pH 2.2, 20 mmol/L potassium phosphate buffer (for the GUS: 5% ACN for 1 min, increased linearly to 50% in 14 min, increased linearly to 90% in 3 min, maintained for 4 min) delivered at a flow rate of 1.5 mL/min. The method for the GUS was 22 minutes. The injection volume was 150 µL in a loop of 20 µL. Detection was performed scanning the 200-400 nm wavelength range. In-house libraries of spectra of drugs, toxic compounds and metabolites were used for compound identification. Table I shows the different quantification methods including the running time and the gradient.

4.1.5. Extraction Recovery Yields

Drug standard prepared in serum were extracted as previously described. For calculation of the recovery yields, the peak area of the compound obtained after the extraction was compared with those of standards prepared in methanol and diluted with the mobile phase to the appropriate concentrations.

4.1.6. Identification and Semi-Quantification

At the completion of the chromatographic procedure, peaks were detected for drugs. Peaks were identified based on their relative retention times. The purity and identity of each peak was assessed by examination of the UV spectra of the peak in comparison to library entries of drugs with similar RTs.

Concentrations of drugs were calculated by comparing the peak area of the analyte and the corresponding standard containing a known amount of the analyte.

4.1.7. Quantification

Quantification methods were developed to determine the extraction recoveries. Table I shows the different quantification methods using all the same mobile phase and the same column described above.

Table I. Quantification Methods by HPLC for more than 100 Substances.

substance group	method	time (min)	phosphate buffer (%)	ACN (%)
Anaesthetics	Anaesthetics1	0	80	20
		9	50	50
Analgesics	Analgesics1	0	90	10
		3-7	60	40
	Analgesics2	0	60	40
		10	40	60
Antidepressants	Antidepressants1	0	75	25
		5-8	50	50
Antidiabetics	Antidiabetics1	0-9	54	46
Antiretroviral	Antiretroviral1	0-3	70	30
		6-12	30	70
Barbiturates	Barbiturats1	0	75	25
		8-10	50	50
Benzodiazepines	Benzodiazepines1	0	75	25
		10	50	50
Betablocking	Betablocking1	0-1	92	8
		5	78	22
		7	75	25
		10-15	70	30
Coumarines	Coumarines1	0-10	50	50
	Coumarines2	0-10	45	55
Diuretics	Diuretics1	0-1	95	5
		10	80	20
	Diuretics2	0-1	70	30
		10	35	65
Drugs of Abuse	Drugsofabuse1	0-1	90	10
		6-10	60	40
Laxatives	Laxatives1	0-4	57	43
		5-8	30	70
Neuroleptics	Neuroleptics1	0	90	10
		8	80	20
	Neuroleptics2	0-2	72	28
		6	50	50
Stimulants	Stimulans1	0-8	80	20
THC	THC1	0-14	40	60

4.2. GENERAL UNKNOWN SCREENING WITH MS

4.2.1. Materials

Test substances obtained from various pharmaceutical companies were of pharmaceutical purity. Organic solvents and reagents were of analytical grade. Acetonitrile and methanol were purchased from Merck (Darmstadt, Germany), ammonium formate from Aldrich (Steinheim, Germany) and formic acid from Fluka (Buchs, Switzerland). Deionised water was generated with a Milli-Q water purification system from Millipore (Kloten, Switzerland). HySphere Resin GP cartridges were purchased from Spark Holland (Emmen, Netherlands).

The Prospekt 2^{TM} unit from Spark Holland (Emmen, Netherlands) consists of an automatic cartridge-exchange module, dual cartridge clamps, solvent delivery unit (SDU) (including a high-pressure dispenser (HPD) and solvents). The solvent delivery unit is used to condition and wash the cartridges with solvents and to transfer the contents of the sample loop of the austosampler into the cartridge. The high-pressure dispenser provides the SPE solvents.

4.2.2. Sample and Buffer Preparation Procedures

Separate stock solutions were prepared in methanol-water (1:1, v/v) at a concentration of 100 μg/mL. Serum standards were prepared by spiking with stock solutions of drug mixtures to make concentrations ranging from 0.005 to 4 μg/mL, resulting in a set of standards with the following concentrations: 0.005, 0.010, 0.025, 0.050, 0.100, 0.250, 0.500, 1.000, 2.000 and 4.000 μg/mL.

d_3-Benzoylecgonine was prepared as internal standard (IS) at a concentration of 5 μg/mL.

The pH 3.0 buffer was prepared with a 10 mmol/L ammonium formate solution, adjusted to the desired pH by appropriate addition of concentrated formic acid.

4.2.3. Extraction Procedure

One ml of serum was acidified by addition of 20 μL concentrated formic acid and 100 μL of internal standard solution were pipetted into each sample.

On-line SPE and elution was performed using the Prospekt 2 system. The HySphere Resin GP cartridge was conditioned with 1 mL methanol (5 mL/min) and with 1 mL water (5 mL/min). The injection volume was 290 µL in a loop of 100 µL. This 100 µL of the serum mixture was loaded on the cartridge. The sorbent was washed with 1 mL water (2 mL/min), and eluted with the mobile phase over 15 minutes. Cartridges were used only one time in order to avoid contamination.

4.2.4. Chromatographic and Mass Spectral Conditions

The chromatographic system consisted of a Rheos 2000 Micro HPLC pump (ThermoFinnigan, Allschwil, Switzerland) and a Midas Symbiosis Autosampler from Spark (Emmen, Netherlands) equipped with a 100 µL loop. A four-channel degasser has been integrated into the Rheos CPS LC System. The LC-MS-MS apparatus was a LCQ Advantage MAX from Thermo Finnigan (Allschwil, Switzerland) equipped with an APCI device operating in the positive and in the negative detection mode.

The chromatographic separation was performed on a CC Nucleodur C18 Gravity 3 µm (4 x 125 mm) with an integrated guard column 3 µm (4 x 8 mm) from Macherey-Nagel (Oensingen, Switzerland). The mobile phase was delivered at a flow rate of 400 µL/min. Each chromatographic run was performed with a binary, linear A/B gradient (Solvent A was 10 mmol/L ammonium formate, pH 3.0. Solvent B was 90% acetonitrile, 10% 10 mmol/L ammonium formate, pH 3.0.). The program was as follows: 0-1 min, 6% B; 1-8 min, 6 to 100% B; 8-20 min 100% B; 20-23 min column equilibration with 6% B. The solvents were degassed.

A data-dependent acquisition was used, generating a full-scan between 80 and 750 amu in the first mode. In the second mode a MS-MS spectrum of the most intense ion of the previous full-scan was performed. Only this high-intensity ion was kept in the ion trap where normalized collision energy is switched on and the resulting fragments were analysed. In the positive mode, normalized collision energy was 40.0%, in the negative mode 35.0%. The mass spectrometer reverted back to the full-scan mode. Dynamic exclusion was enabled. Thirty seconds refractory period was applied to the last selected ion.

The following APCI inlet conditions were applied. The heated vaporizer was kept at 465 °C. Both the sheath gas and the auxiliary gas were nitrogen set at 60 and 15 relative units, respectively. The capillary entrance to the ion trap was at an offset of 28 V in the positive mode, -4 V in the negative mode and was maintained at 220° C. The corona current was 5 µA. Table II shows the data dependent and global data dependent settings.

Table II. Data Dependent and Global Data Dependent Settings.

Data Dependent Settings	
Default Charge State	1
Default Isolation Width	4.0
Normalized Collision Energy (%)	40.0 resp. 35.0
Min. Signal Required	20000
Global Data Dependent Settings	
Exclusion Mass Width	0.5
Reject Mass Width	1.0
Dynamic Exclusion	enabled
Repeat Count	1
Repeat Duration	0.5
Exclusion List Size	25
Exclusion Duration	0.5
Exclusion Mass Width	0.5

4.2.5. Mass Spectral Library Building Conditions

Standard solutions were prepared in methanol-water (1:1, v/v) at a concentration of 1-2 µg/mL. Two mass spectral libraries were created, one for each ionization mode, by injecting 20 µL of these solutions directly without HPLC separation into the MS system. The MS-MS spectra were obtained at 40.0% normalized collision energy in the positive mode and 35.0% normalized collision energy in the negative mode. The obtained MS-MS spectra were added to the library.

Relative retention data were acquired by actual LC-MS (-MS) analysis running a mixture of each compound with the internal standard.

A library was constructed to include per spectra, the name of the compound, the molecular formula, and its protonated molecular ion, together with the relative retention time. The mass spectral library comprises a total of more than 400 spectra of more than 350 compounds. For a semi-quantitative procedure peak areas of the compounds (at a concentration of 1 μg/mL) were recorded and attached to the library.

Obtained MS-MS data from a chromatographic run were compared to the MS-MS library using the NIST Mass Spectral Program 2.0 from ThermoFinnigan. A computer program (XcLibraryScreening) was created to automate the searching process and to include the RRT and the molecular ion in the identification of unknown compounds.

5. RESULTS AND DISCUSSION

5.1. SAMPLE PREPARATION AND CHROMATIC CONDITIONS FOR A GUS

The sample preparation must be suitable for various compounds in a large polarity range (acidic, neutral and basic). Thus, it requires a nonclass-specific extraction procedure able to isolate the widest range of relevant molecules from biological matrices. Such procedures may involve a single liquid-liquid extraction, two liquid-liquid extraction in parallel (one for acidic and neutral and one for basic compounds) (51, 54-56), solid-phase extraction with classical hydrophobic phase (49, 57), or mixed-mode phase SPE (49, 57-60).

SPE was chosen as extraction technique. The SPE mechanism is more selective than liquid-liquid extraction. SPE has a number of advantages over liquid-liquid extractions such as cleaner extracts, increased selectivity for the compounds of interest, and smaller volumes of solvent may be used for extraction. SPE takes less time, and the procedure can be automated (11).

A polymer-based sorbent was selected because it is stable over a larger pH range and does not have secondary (unbounded hydroxy silanol) groups compared to classical hydrophobic phase.

A mixed-mode phase involves both reversed-phase retention and cation-exchange properties. The column elution requires two steps. First the acidic and neutral substances were eluted with methanol. In the second step the basic substances were eluted with a methanol/NH_4OH mixture. The acid-neutral and the basic fractions were mixed before injection in the HPLC-DAD system in order to save time.

Absolute recoveries were measured for the polymer (Oasis HLB) and the mixed-mode-based sorbent (Oasis MCX). The Oasis HLB column showed the better extraction recoveries for most of the tested substances than the Oasis MCX column.

5.1.1. Sample Preparation and Chromatic Conditions with HPLC

Before loading a sample on the cartridge, 20 μL of orthophoshoric acid (85%) (or 20 μL of formic acid in the LC-MS method) was added to the sample. The addition of acids influenced the protein-binding. The pH was lowered resulting in a disruption of the drug-protein interaction yielding better recoveries. Above all, highly-protein bound drugs showed a much better recovery. For example the extraction recovery for torasemide was improved from 49% to 86%.

For each compound of the library, absolute recovery was measured at least two times by extracting and assaying drug-free serum spiked with the drugs tested, then comparing the peaks areas of these extracted standards with those of methanolic standards at the same concentration.

Of the tested drugs, 94 of 100 were successfully extracted. A successful extraction is defined as a recovery of more than 50%. The extraction of more than 50% of the tested drugs results in a recovery of more than 90%. Fig. 2 shows the absolute recovery of the 100 tested compounds.

This method proved to extract a large range of drugs. Acid, neutrals as well as basic drugs were extracted in a satisfying way. Only 6 of the hundred tested drugs showed an extraction recovery of less than 50%. These drugs are bromadiolone (extraction recovery 31%), chlorophacinone (8%), delta-8-THC (34%), delta-9-THC (47%), emodine (40%) and rhein (19%). They are all hydrophobic and mostly of them acidic substances. 1 mL of methanol was not sufficient to elute these compounds in a satisfying way (≥ 50%) from the column. Improvement of the extraction recovery for these substances could be achieved by using a higher volume of methanol, a stronger solvent than methanol or another column. Acidic substances were not charged at the present pH because of the addition of the ortho-phosphoric acid. Also a change of the pH could improve the extraction recovery.

The extraction also proved to be reproducible. Table III shows the extraction recovery and coefficient of variation of the 100 tested compounds. The coefficient of variation was always less than 15% for all of the compounds with an extraction recovery of more than 50%. The only exception was secobarbital showing a coefficient of variation of 16.2%.

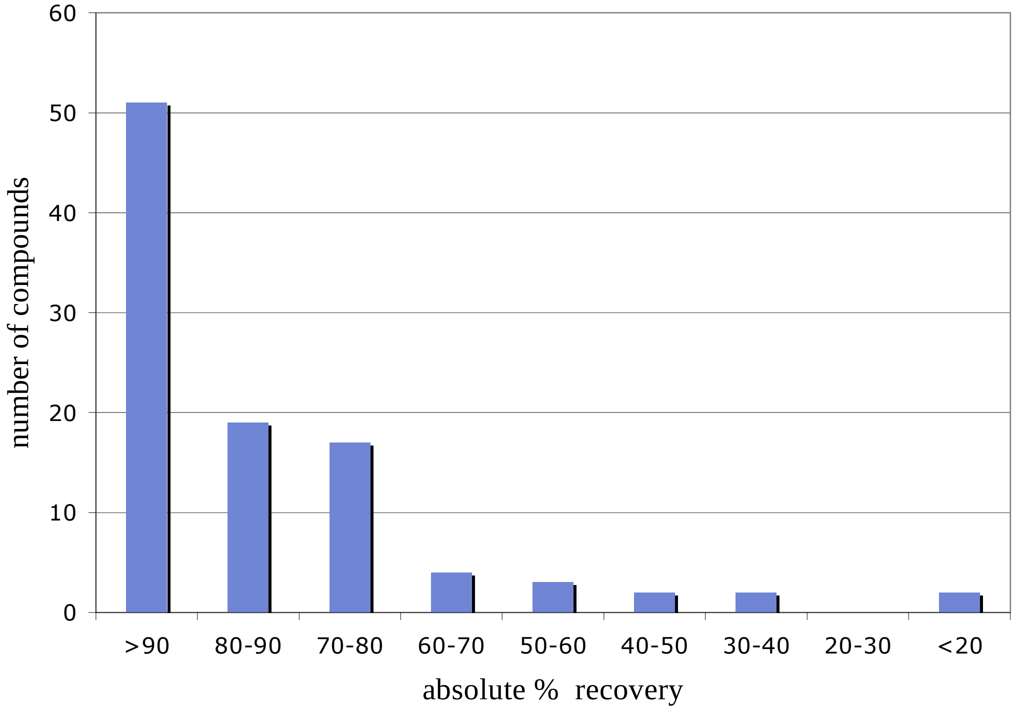


Fig. 2. Absolute % recovery of the 100 tested compounds with HLB Oasis columns.

Gradient studies were performed to achieve a fast elution ramp without losing too much sensitivity and resolution. It was decided that a 5 to 50% acetonitrile gradient over 14 minutes, and a 50 to 90% acetonitrile gradient over 3 minutes would be suitable for this method. A slow gradient was chosen for the first gradient because most of the compounds were eluted in a short time from the column with 50% of acetonitrile. These compounds showed a retention time of less than 15 minutes. The other substances were eluted with the fast 50-90% acetonitrile gradient. Analytical results can be interpreted after 22 minutes. This is an acceptable turnaround time for the analysis of emergency drug screening results. In Table III the retention time of the 100 substances are listed.

Table III. LOD, Recoveries of Drugs and their Coefficient of Variation Using the HLB Column and Recorded Retention Time of 100 Compounds.

Compounds	LOD (ng/mL)	Recovery (%)	CV (%)	Retention time (min)
6-acetylmorphine	1’000	105	3.5	6.20
acebutolol	1’000	85	2.8	7.55
acenocoumarol	250	85	4.0	14.99
acetaminophen	500	99	0.9	3.97
acetazolamide	500	95	2.0	3.57
acetylsalicylic acid	1’000	97	0.2	8.53
aloeemodine	500	60	13.2	13.76
alprazolam	1’000	66	14.7	13.32
alprenolol	2’000	93	2.4	10.57
amiloride	250	95	0.7	4.11
amitryptiline	500	75	4.3	13.37
amobarbital	5’000	83	5.1	11.43
amprenavir	1’000	54	3.0	14.43
atenolol	2’000	85	1.4	5.10
benzoylecgonine	1’000	94	1.7	7.42
bisacodyl	500	64	0.5	12.94
brallobarbital	5’000	106	1.4	9.37
bromadiolone	<ER	31	16.4	18.39
bupivacaine	1’000	101	0.8	10.53
butalbital	2’000	89	0.4	10.25
cannabidiol	1’000	72	8.0	19.25
cannabinol	500	51	13.0	19.82
canrenone	500	93	4.4	15.10
carbromal	5’000	72	5.4	10.97
chlordiazepoxide	250	96	2.0	9.21
chlorophacinone	<ER	8	56.8	18.85
chlorthalidone	1’000	85	3.3	9.20
cinchocaine	1’000	97	0.5	12.80
citalopram	2’000	79	2.4	12.09
cocaethylene	500	100	0.7	11.15
cocaine	1’000	92	4.7	9.37
codeine	1’000	85	2.7	5.05
coumachlor	500	76	3.9	15.93
coumaphos	1’000	72	6.2	18.00
coumatetralyl	250	78	1.9	16.11
crimidine	500	88	1.7	6.57
delta-8-THC	>2’000	34	18.0	20.21
delta-9-THC	2’000	47	12.0	20.16
diazepam	2’000	78	3.3	13.83
diclofenac	1’000	92	1.4	15.81
dihydrocodeine	1’000	94	4.0	4.67
efavirenz	1’000	80	6.0	16.86
emodine	1’000	40	12.9	16.02
ephedrine	1’000	89	2.4	3.74
flupenthixol	1’000	95	5.2	12.53

Table III (continued). LOD, Recoveries of Drugs and their Coefficient of Variation Using the HLB Column and Recorded Retention Time of 100 Compounds.

Compounds	**LOD (ng/mL)**	**% Recovery**	**CV (%)**	**Retention time (min)**
furosemide	1’000	66	1.8	12.07
glibenclamide	1’000	97	1.7	16.23
glibornuride	1’000	106	7.0	15.23
gliclazide	1’000	75	8.6	14.51
hydrochlorothiazide	500	83	0.2	5.40
hydrocodone	1’000	86	3.7	6.36
hydromorphone	500	92	3.8	3.00
ibuprofen	1’000	74	1.8	15.65
imipramine	2000	94	7.4	13.02
indinavir	1’000	92	1.9	10.72
levomepromazine	500	98	0.0	12.85
lidocaine	1’000	93	4.7	7.38
lopinavir	2’000	56	8.9	16.63
lorazepam	1’000	90	5.9	12.43
mefenamic acid	1’000	78	7.1	16.65
mepivacaine	1’000	99	5.1	7.39
methylphenidate	2’000	99	1.3	8.88
metoprolol	1’000	96	3.6	7.83
morphine	2’000	88	4.7	3.49
nadolol	5’000	85	8.8	5.80
nalbuphine	500	91	1.0	6.70
naproxen	500	89	3.4	13.82
nelfinavir	1’000	91	2.7	14.45
nevirapine	500	93	7.4	9.76
norcodeine	500	95	0.9	4.60
olanzapine	250	99	1.6	6.26
oxprenolol	2’000	100	6.9	9.09
pentobarbital	5’000	77	1.0	11.29
phenobarbital	2’000	95	2.2	9.46
phenolphthalein	500	79	1.7	13.19
phenprocoumon	500	88	6.3	15.97
pindolol	2’000	76	10.0	7.50
propranolol	1’000	95	5.8	10.43
propyphenazone	500	93	1.8	12.07
pseudoephedrine	1’000	92	2.7	3.74
quetiapine	500	90	5.8	9.74
rhein	<ER	19	5.0	14.10
ritalinic acid	1’000	100	1.6	7.15
ritonavir	1’000	96	4.8	16.34
salicylic acid	500	97	1.9	9.59
saquinavir	1’000	94	2.3	14.41
secobarbital	5’000	74	16.2	12.07
sertraline	>2’000	80	3.0	13.74
sotalol	1’000	89	4.1	4.50
spironolactone	1’000	79	5.6	15.67

Table III (continued). LOD, Recoveries of Drugs and their Coefficient of Variation Using the HLB Column and Recorded Retention Time of 100 Compounds.

Compounds	LOD (ng/mL)	% Recovery	CV (%)	Retention time (min)
thiopental	250	74	1.1	13.77
thioridazine	500	95	4.0	14.35
timolol	1’000	93	4.7	7.71
tolbutamide	500	98	1.7	13.41
torasemide	500	86	1.1	10.64
trimipramine	1’000	94	4.4	13.47
tubocurarine	500	98	5.7	8.71
venlafaxine	1’000	90	9.3	9.96
warfarin	1’000	95	1.7	14.73
zuclopenthixol	1’000	90	8.8	11.62

5.1.2. Sample Preparation and Chromatographic Conditions with LC-MS

On-line SPE was chosen as an extraction technique because this procedure is universal, rapid and can be automated. This method is becoming popular in bioanalytical analysis (61). The Prospekt solid phase extraction can be linked to the APCI ion source of the LC-MS-MS instrument (62). The system couples and automates sample extraction and instrumental analysis. Benefits of this technology include improved precision of all extraction steps. This method has a time saving advantage compared to other techniques because vaporizing the sample extract is not necessary. In addition, the procedure presented in this study extracts acidic, neutral as well as basic drugs.

The chromatographic conditions (column and mobile phase) must be chosen in an appropriate way. The most polar must be retained and the most lipophilic compounds must be eluted. The mobile phases must be compatible with API sources.

Most of the HPLC-DAD applications use non-volatile buffers such as phosphate and borate buffers. These non-volatile buffers cause problems in combination with MS and should be avoided. They are blocking the capillary in the probe, and are causing salt buildup on the spray head and thus compromise the integrity of the spray. Volatile buffers like ammonium acetate, ammonium formate, acetic acid and ammonium carbonate buffers can be used instead. Ammonium formate was chosen because of its suitability as a buffer at a pH of 3. Ammonium is necessary for a better elution of the compounds in combination with the Prospekt and often avoids the tailing of peaks.

An appropriate column has to be chosen in combination with the Prospekt. The column has to retain all the compounds stronger than the extraction column of the Prospekt to obtain a satisfying chromatography. A C_{18} Nucleodur Gravity column was chosen because of its capability to be linked with the Prospekt, its stability within a large pH range and its robustness.

The separation of the drugs was carried out under acidic conditions (pH = 3) in order to limit secondary interaction on the free silanol groups of the C_{18} column. The first peak eluted at 5.9 minutes (morphine) and the last at 18.4 minutes (delta-8-THC). The absolute retention times are shown in Table IV. Separation of substances is necessary to detect low-level analytes. Ionization of mobile phase components (acetonitrile, ammonium formate) and endogenous compounds is the main source of background noise. Contamination of the mass spectra by these compounds potentially hampers the identification of low concentrations of analytes. The screening of a sample can be performed in less than one hour with a chromatographic run taking 23 minutes for each mode (positive and negative), which is an acceptable analytical time for a GUS (including library search and interpretation).

APCI and ESI were compared with each other in order to examine with which ion source the higher signals were detected. More than hundred compounds were investigated in aqueous solutions.

In summary, higher signals were detected with APCI for hydrophobic substances like cannabinoids or coumarines and acidic drugs. Better results were obtained with ESI for basic drugs such as neuroleptics and antidepressants. Basic drugs are positively charged at a pH of 3.0 (pH of the mobile phase).

These results are not astonishing. The relation of the molecular mass range and the polarity of analytes that can be analyzed by GC-MS, APCI LC-MS and ESI LC-MS techniques are sketched in Fig. 3. APCI allows sensitive determination of analytes with moderate polarity and molecular mass. ESI enables the better determination of analytes of high molecular mass (up to several hundred units such as peptides or proteins) and high polarity.

Table IV. Recorded LC Retention Times of 87 Compounds.

Compounds	Retention time (min)	Compounds	Retention time (min)
morphine	5.89	imipramine	8.40
amiloride	6.10	phenobarbital	8.45
atenolol	6.10	amitryptiline	8.48
hydromorphone	6.16	canrenone	8.48
sotalol	6.25	trimipramine	8.55
codeine	6.30	brallobarbital	8.56
dihydrocodeine	6.42	nelfinavir	8.57
norcodeine	6.42	sertraline	8.58
6-acetylmorphine	6.51	zuclopenthixol	8.58
acetaminophen	6.53	saquinavir	8.61
olanzapine	6.53	thioridazine	8.93
hydrocodone	6.55	crimidine	8.97
pseudoephedrine	6.55	furosemide	8.97
ephedrine	6.56	phenolphthalein	9.08
nadolol	6.60	alprazolam	9.16
tubocurarine	6.67	lorazepam	9.28
nalbuphine	6.76	propyphenazone	9.67
benzoylecgonine	6.77	amprenavir	9.80
acetazolamide	6.81	tolbutamide	9.80
ritalinic acid	6.86	rhein	10.30
pindolol	6.89	aloeemodine	10.34
mepivacaine	7.00	acenocoumarol	10.40
acebutolol	7.05	gliclazide	10.49
timolol	7.05	warfarin	10.53
lidocaine	7.06	glibornuride	10.57
metoprolol	7.19	bisacodyl	10.59
cocaine	7.27	glibenclamide	10.73
oxprenolol	7.32	ritonavir	10.82
hydrochlorothiazide	7.43	diazepam	10.87
venlafaxine	7.45	lopinavir	10.89
cocaethylene	7.50	phenprocoumon	10.91
bupivacaine	7.76	diclofenac	11.11
propranolol	7.76	coumachlor	11.12
alprenolol	7.79	coumatetralyl	11.17
chlorthalidone	7.80	efavirenz	11.58
quetiapine	7.88	emodine	11.65
indinavir	7.92	mefenamic acid	12.05
torasemide	7.99	bromadiolone	12.85
citalopram	8.02	chlorophacinone	13.78
levomepromazine	8.02	cannabidiol	14.15
nevirapine	8.08	cannabinol	16.58
chlordiazepoxide	8.17	delta-9-THC	18.23
flupenthixol	8.33	delta-8-THC	18.44
cinchocaine	8.39		

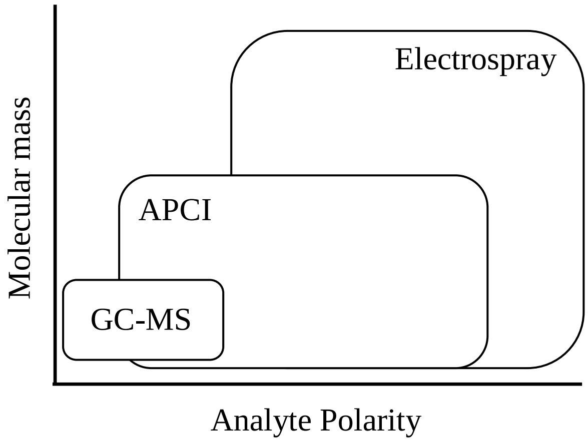


Fig. 3. Relation of the molecular mass range and the polarity of analytes analyzable by GC-MS and LC-MS interface techniques (APCI and ESI).

This was confirmed by Bogusz et al. They showed that between these two ionization sources, atmospheric pressure chemical ionization appeared more universal and assured generally higher sensitivity. Only in the case of very polar drugs (e.g. psilocin or psilocybin) electrospray ionization was more sensitive (63).

For a GUS it is not necessary to detect high molecular mass. Most of drugs have masses under 500 amu and are moderately polar. But at an acidic pH (required in this method) a lot of compounds are positively charged and become more polar. None of the two ion sources showed a large benefit in this examination.

APCI was preferred to electrospray ionization in order to reduce ion suppression. This phenomenon affects the amount of charged ion in the gas phase that reaches the detector. Although ion suppression can have effects on both electrospray ionization and atmospheric pressure chemical ionization, evidence indicates that the electrospray interface is more impacted. Experiments have shown that with the same extraction procedure and method ESI was more affected by ion suppression than APCI. Serums or aqueous solutions were spiked with the same amount of a compound. Ionization with ESI resulted in a large variability between the matrix specimen and the aqueous solution. The signal of the analyte in serum was mostly decreased. The extent of the ion suppression is not being predictable. In a GUS ion suppression is responsible for higher LODs. In the worst case ion suppression can result in false negative results.

5.2. LIBRARY SCREENING AND QUANTIFICATION

5.2.1. Identification with HPLC

The present method allows easy and rapid identification of 100 substances belonging to different pharmacological classes. The LOD of all the substances are listed in Table III.

UV spectra and retention time databases were established by spiking aqueous with pure drugs for the identification of drugs extracted from serum. Bogusz et al showed that the co-extracted biological matrix did not exert any influence on the chromatographic behaviour of drugs analyzed by means of gradient HPLC (28).

Serum samples spiked with decreasing concentrations of the tested drugs were analyzed in order to determine LODs. Each concentration was extracted and analysed two times. The LOD was set at the lowest concentration where the signal of the compound was three times higher than the background noise and the spectral similarity was above the cut-off (> 0.9).

Identification of drugs with diode array detection and a spectrum library are affected by the presence of co-extracted matrix substance. These compounds can co-elute at the same time as toxicologically relevant substances and overlap the corresponding UV spectra. The purity of the extract is therefore important for a successful HPLC-DAD identification of a drug even at small concentrations.

In order to increase the specificity of the method, each drug of the library is also characterized by its retention time. The retention time has to be in a time window of ± 0.5 min. Identification errors require coincidence of both RTs and UV spectra.

The LOD was set at the lowest concentrations where both samples fulfilled the mentioned requirements (spectra similarity and retention time). The limit of detection (LOD) for the majority of the tested drugs (76%) was ≤ 1’000 ng/mL.

Shifts of the retention time of compounds are characteristics of changes in the molarity or pH of the mobile phase or ageing of the column. Also two columns of the same brand can cause a shift of the retention time.

The use of retention times relative to an internal standard reduces the influence of the altering of the column or changing the column. Relative retention time is more reproducible than the retention time. Bogusz et al showed that relative retention time data can be used interlaboratory (64, 65). With this procedure the retention time can be corrected and is less affected by the mentioned influences.

5.2.2. A Semi-Quantitative Procedure with HPLC

Not only the identification of compounds is important, also the determination of the concentration is crucial in a GUS to estimate the severity of the intoxication. A semi-quantitative procedure was performed. Drug-free serum spiked with the drugs tested were extracted and analyzed. Peak areas of extracted serum sample were compared to peak areas of methanolic standards of the database considering the extraction recovery. Results are shown in Table IV. The measured concentration of 72 of totally 94 substances was within 20%. These results indicated the possibility to estimate the drug concentration with this procedure.

Co-extracted matrix substances affect the quantitation of drugs eluting at the same time. This is one of the major causes of errors in chromatographic analysis. An erroneous result can be caused by a coeluting matrix substance that mimics the spectrum of a compound listed in Table V or interfere with the detection of a part of a peak.

The basis of the assay was to provide a means to identify a drug or poison and an estimate of its concentration. More accurate quantitation was then conducted using the quantification method.

Table V. Extraction rate of spiked serum samples compared to methanolic standard concentrations.

Drug	Measured (µg/mL)	Difference (%)	Drug	Measured (µg/mL)	Difference (%)
6-acetylmorphine	0.99±0.28	-0.8%	hydrocodone	0.70±0.12	-29.7%
acebutolol	2.22±0.33	10.9%	hydromorphone	0.98±0.16	-2.4%
acenocoumarol	0.57±0.01	14.8%	ibuprofen	6.54±0.08	30.8%
acetaminophen	4.19±0.30	-16.3%	imipramine	1.80±0.20	-9.8%
acetazolamide	0.95±0.00	-4.8%	indinavir	0.94±0.03	-5.7%
acetylsalicylic acid	1.16±0.16	-41.9%	levomepromazine	0.96±0.01	-4.1%
aloeemodine	2.02±0.10	0.8%	lidocaine	0.88±0.11	-11.7%
alprazolam	0.53±0.11	-46.5%	lopinavir	1.57±0.09	-21.3%
alprenolol	1.85±0.21	-7.4%	lorazepam	1.09±0.02	9.2%
amiloride	0.71±0.04	-29.0%	mefenamic acid	1.42±0.02	-28.9%
amitryptiline	1.46±0.32	45.8%	mepivacaine	1.13±0.02	12.9%
amobarbital	2.28±0.40	-54.3%	methylphenidate	1.93±0.05	-3.5%
amprenavir	1.22±0.05	21.5%	metoprolol	0.92±0.11	-7.9%
atenolol	2.05±0.05	2.5%	morphine	1.79±0.18	-10.3%
benzoylecgonine	1.17±0.00	17.5%	nadolol	5.28±0.62	5.7%
bisacodyl	1.44±0.00	44.3%	nalbuphine	1.12±0.27	12.2%
brallobarbital	4.67±0.07	-6.7%	naproxen	5.71±0.12	14.3%
bupivacaine	0.80±0.04	-19.8%	nelfinavir	1.13±0.03	12.7%
butalbital	1.94±0.06	-3.0%	nevirapine	0.86±0.08	-14.5%
cannabidiol	0.54±0.01	-46.4%	norcodeine	0.89±0.01	-10.9%
cannabinol	1.18±0.08	-40.9%	olanzapine	0.69±0.13	-30.7%
canrenone	0.99±0.02	-1.2%	oxprenolol	1.99±0.31	-0.3%
carbromal	6.29±0.11	25.7%	pentobarbital	4.66±0.11	16.6%
chlordiazepoxide	0.46±0.01	-7.8%	phenobarbital	1.73±0.08	-13.7%
chlorthalidone	1.01±0.15	1.2%	phenolphthalein	1.74±0.02	-13.0%
cinchocaine	1.05±0.16	5.4%	phenprocoumon	0.47±0.04	-5.1%
citalopram	2.00±0.03	-0.1%	pindolol	1.17±0.40	-41.3%
cocaethylene	1.02±0.01	2.2%	propranolol	1.04±0.00	4.3%
cocaine	0.97±0.06	-2.6%	propyphenazone	4.79±0.04	-4.3%
codeine	1.14±0.01	14.3%	pseudoephedrine	0.81±0.25	-19.2%
coumachlor	0.99±0.08	-0.8%	quetiapine	1.07±0.22	6.9%
coumaphos	1.09±0.06	8.9%	ritalinic acid	0.79±0.03	-20.7%
coumatetralyl	0.97±0.06	-2.9%	ritonavir	0.52±0.00	-48.4%
crimidine	1.01±0.11	0.8%	salicylic acid	4.27±0.13	-14.6%
diazepam	1.74±0.28	-13.1%	saquinavir	0.83±0.02	-17.3%
diclofenac	4.29±0.36	-14.3%	secobarbital	4.28±0.74	-14.4%
dihydrocodeine	0.77±0.07	-22.5%	sertraline	4.15±0.36	38.3%
efavirenz	1.04±0.13	3.9%	sotalol	1.86±0.25	-7.2%
ephedrine	0.92±0.01	-8.2%	spironolactone	0.69±0.02	-31.4%
flupenthixol	1.04±0.13	-16.0%	thiopental	0.58±0.00	16.7%
furosemide	1.00±0.05	16.4%	thioridazine	1.12±0.21	12.2%
glibenclamide	0.92±0.01	-0.5%	timolol	1.03±0.01	3.2%
glibornuride	0.84±0.06	-25.2%	tolbutamide	0.81±0.04	-18.7%
gliclazide	1.16±0.21	-10.4%	torasemide	1.09±0.05	8.8%
hydrochlorothiazide	1.00±0.13	13.0%	trimipramine	0.86±0.18	-13.6%

Table V (continued). Testing of the Semi-Quantitative General Unknown Screening procedure.

Drug	Measured (µg/mL)	Difference (%)	Drug	Measured (µg/mL)	Difference (%)
tubocurarine	1.19±0.18	19.3%	warfarin	0.80±0.20	-20.3%
venlafaxine	1.14±0.09	13.9%	zuclopenthixol	0.88±0.21	-12.3%

5.2.3. Method Development

MS-MS data results higher specificity and selectivity and more structural information for the identification of an unknown substance. First the unknown compound has to be detected. In the second step a product-ion scan has to be applied. This procedure is not compatibile for a GUS with the classic MRM mode. Mass spectra recorded with in-source CID don’t result from a single parent ion resulting in low specificity and selectivity.

The method of choice was a data-dependent acquisition procedure. In the first step (so-called "survey scan") the MS-MS instrument is operated in the full-scan single-mass mode. In the second mode the most intense ion above a predefined threshold was selected. This ion was fragmented in the collision cell. The resulting fragments were analyzed in the scan mode.

A refractory period was set to the last ion selected for 30 seconds. Less intense ions of compounds eluting at the same time would not be detected without a refractory period. A refractory period longer than 30 seconds can result in a loss of identification of a compound with the same molecular mass ion. With a shorter refractory period the method can fail to detect substances eluting at the same time. After a short time the instrument switched back to the initial conditions.

Mass spectra data were established by spiking methanol-water (1:1; v/v) with pure drugs for the identification of drugs extracted from serum. Mass spectra were recorded in the negative as well as in the positive mode; because some substances can only be detected in one mode or they have different LOD for both modes. For example morphine was better detected with the positive mode, bromadiolone better detected in the negative mode. For both modes a library was created. In best cases, substances with positive and negative spectra can be identified in both modes with the respective libraries. So results from both libraries confirm each other in most cases.

The MS-MS spectra were obtained at 40.0% normalized collision energy in the positive mode and 35.0% normalized collision energy in the negative mode. Examples of product ion mass spectra are shown in Fig. 4 and Fig. 5. The MS-MS spectrum of cocaine in the positive and the spectrum of saquinavir in the negative mode are presented. The normalized collision energies of 40.0% in the positive and 35.0% in the negative mode were chosen in order to obtain fragmentation of the compounds. A decrease of the normalized collision energy would yield less fragmentation. Applying higher normalized collision energy would result in much lower peak intensities of the fragments.

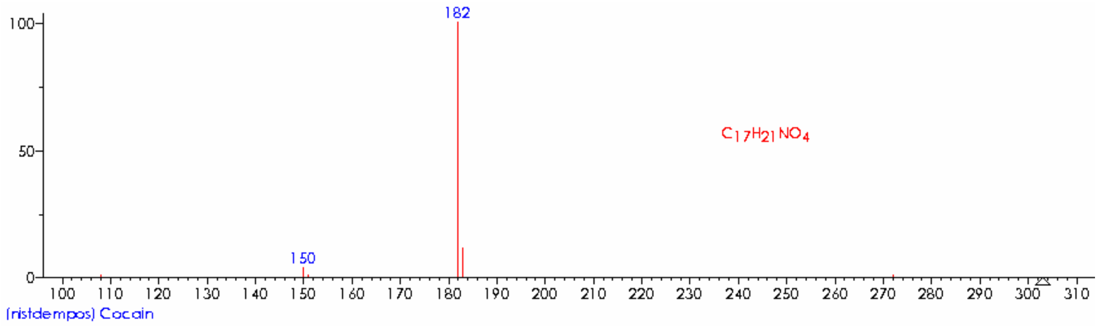


Fig. 4. MS-MS spectrum of cocaine obtained in the positive mode with normalized collision energy of 40.0%.

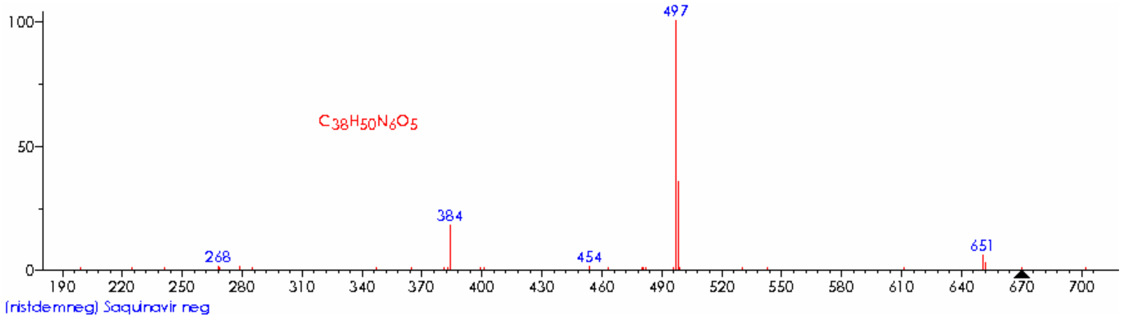


Fig. 5. MS-MS spectrum of saquinavir obtained in the negative mode with normalized collision energy of 35.0%.

The relative retention time data were gathered by running a mixture of each compound with the internal standard by an actual LC-MS (-MS) analysis.

A library was constructed to include per spectra, the name of the compound, the molecular formula, and its protonated molecular ion, together with its relative retention time. This mass spectral library comprises spectra of drugs and metabolites from a large diversity of substance classes. With this procedure acidic, neutral as well as basic drugs can be detected and identified.

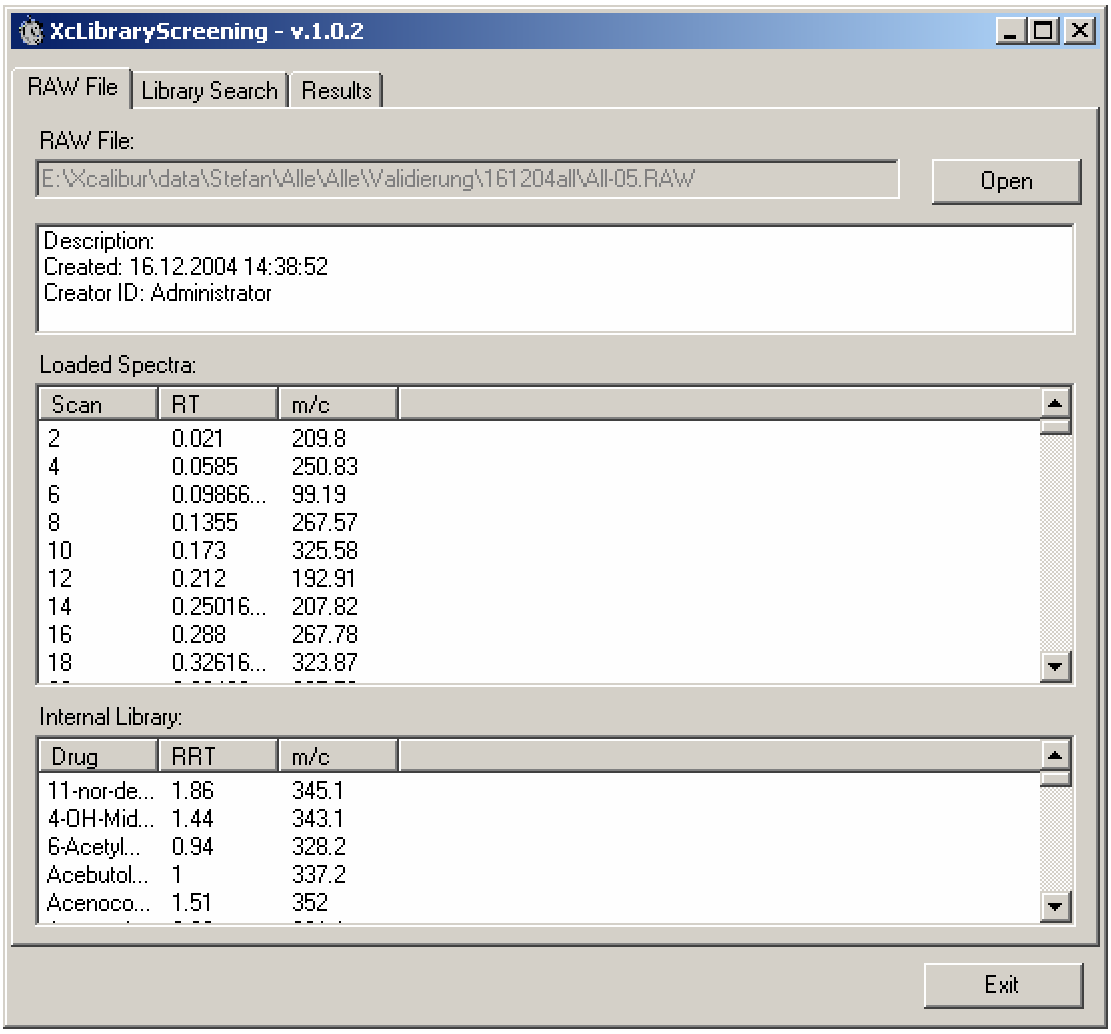
5.2.4. Automatic Library Searching with LC-MS

A small application program was developed for the automated identification of unknown compounds with LC-MS.

In order to identify unknown compounds in a serum sample a chromatographic run was performed in each mode. In the next step the developed program compared each recorded MS-MS spectrum to those in the library from the Xcalibur software. With this program the number of best hits that the unknown spectrum should be compared to can be specified. In the procedure described in this study the ten best hits were chosen.

In the presented example a run was loaded of a serum sample spiked with 5 substances (Fig. 6). The run was performed in the positive mode. Phenolphthalein, gliclazide, bisacodyl, glibornuride and glibenclamide were added to a serum sample at a concentration of 1 µg/mL.

The similarity between the library spectra and the unknown spectra is characterized by the match factor and the reverse match factor. The match factor indicates the correlation between the unknown spectrum and the library spectrum (presence and relative intensities of mass-to-charge ratios). The reverse match factor indicates an inverse search. The presence and the relative intensity of the ions of the library spectrum are compared to those of the unknown spectrum. This parameter ignores the ions present in the unknown spectrum if absent in the reference spectrum.



RAW File: E:\Xcalibur\data\Stefan\Alle\Alle\Validierung\161204all\All-05.RAW

Description:
Created: 16.12.2004 14:38:52
Creator ID: Administrator

Loaded Spectra:

Scan	RT	m/c
2	0.021	209.8
4	0.0585	250.83
6	0.09866...	99.19
8	0.1355	267.57
10	0.173	325.58
12	0.212	192.91
14	0.25016...	207.82
16	0.288	267.78
18	0.32616...	323.87

Internal Library:

Drug	RRT	m/c
11-nor-de...	1.86	345.1
4-OH-Mid...	1.44	343.1
6-Acetyl...	0.94	328.2
Acebutol...	1	337.2
Acenoco...	1.51	352

Fig. 6. All MS-MS spectra were loaded from a run. They were compared to the spectra in the library.

The match factor and the inverse match factor range between 0 and 1’000 with 0 indicating no similarity and 1’000 indicating perfect similarity. In our procedure for both factors the threshold was set at 400. With this threshold parameter the best results were obtained. It is also possible to set another threshold. A higher threshold can result in a higher LOD and in a more specific result. A lower threshold can result in false negatives but also in a lower LOD.

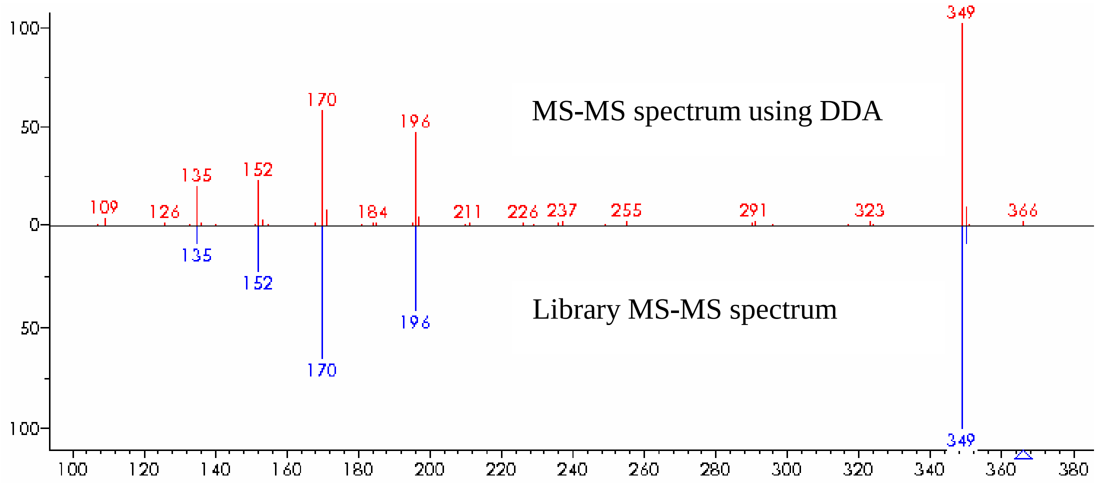


Fig. 7. Comparison of the positive MS-MS spectrum of glibornurid in a serum sample using DDA to the MS-MS spectrum of the library.

Each product ion mass spectrum was subjected to an automated library searching routine compared to the library spectra. Fig. 7 shows the MS-MS spectrum of gliclazide from spiked serum (above in red colour) obtained with this procedure compared to the MS-MS spectrum of the library (below in blue colour). The match factor of the presented mass spectra was 889, the reverse match factor was 989.

Compound identification took in account the mass-to-charge ratio of the unknown compound selected before fragmentation. This mass-to-charge ratio has to be within ± 2 m/z of the reference mass-to charge ratio recorded in the library. The width of the mass-to-charge ratio window was chosen to include the isotopes of the compound.

The pseudomolecular ion (usually, protonated in the positive mode, deprotonated in the negative mode) and its fragments were obtained and compared to references in the library. Each MS-MS spectrum recorded is derived from one single mass-to-charge ratio (representing the most intense ion of the previous full scan).

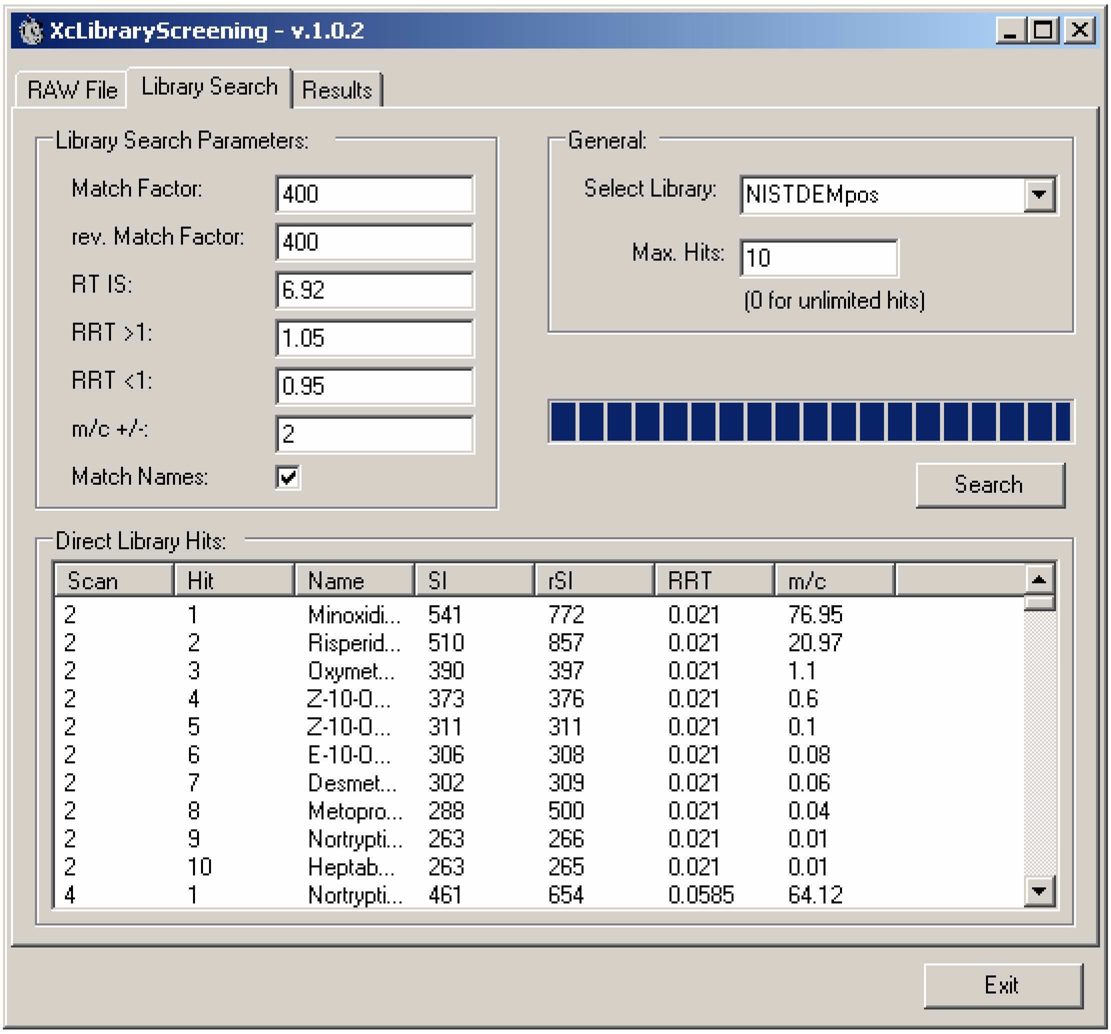
Other authors used collision-induced dissociation at different voltages to obtain the same information. Mass spectra were acquired by continuously switching between a low and a high orifice voltage throughout the run to obtain both protonated molecular ion (low-voltage scan) and mass spectral fragments (high voltage scan) from the CID in the ion source. The low-voltage spectrum was dominated by the pseudomolecular ion, whereas the high-voltage spectrum contained mass fragment ions. Mass spectra from the two different voltage scans were then summed to produce a mass spectrum for each compound in the library in order to maximize the information. With the procedure presented in this study it is not necessary to switch between different orifice voltages.

RRT was also included in the identification procedure. The RRT of the unknown compound has to be within ± 5%. This large time-window was chosen because the MS-MS spectra can be obtained during the whole peak width and the refractory period was set at 30 s. The RT of the IS was registered in the positive mode with a value of approximately 6.9 min. Variations in RT occur when using different lots of columns with the same absorption material.

Only if all the parameters were within the fixed areas a hit was reported. In summary the match factor and the reverse match factor had to be above 400, the mass-to-charge had to be ± 2 m/z and the RRT had to be within 5% (Fig. 8). Each MS-MS spectrum, which fulfilled these conditions, was reported.

The report consists of the different hits with their names, together with the match factor and reverse match factor of the comparison with the library spectrum, relative retention time and mass-to-charge in this run and the one in the library.

The new program automatically releases a report, which consists of the different hits with the substance names, together with the match factor, the reverse match factor, the RRTs and mass-to-charge relations compared to the ones in the library.



Library Search Parameters:

Match Factor: 400
rev. Match Factor: 400
RT IS: 6.92
RRT >1: 1.05
RRT <1: 0.95
m/c +/-: 2
Match Names: ☑

General:

Select Library: NISTDEMpos
Max. Hits: 10
(0 for unlimited hits)

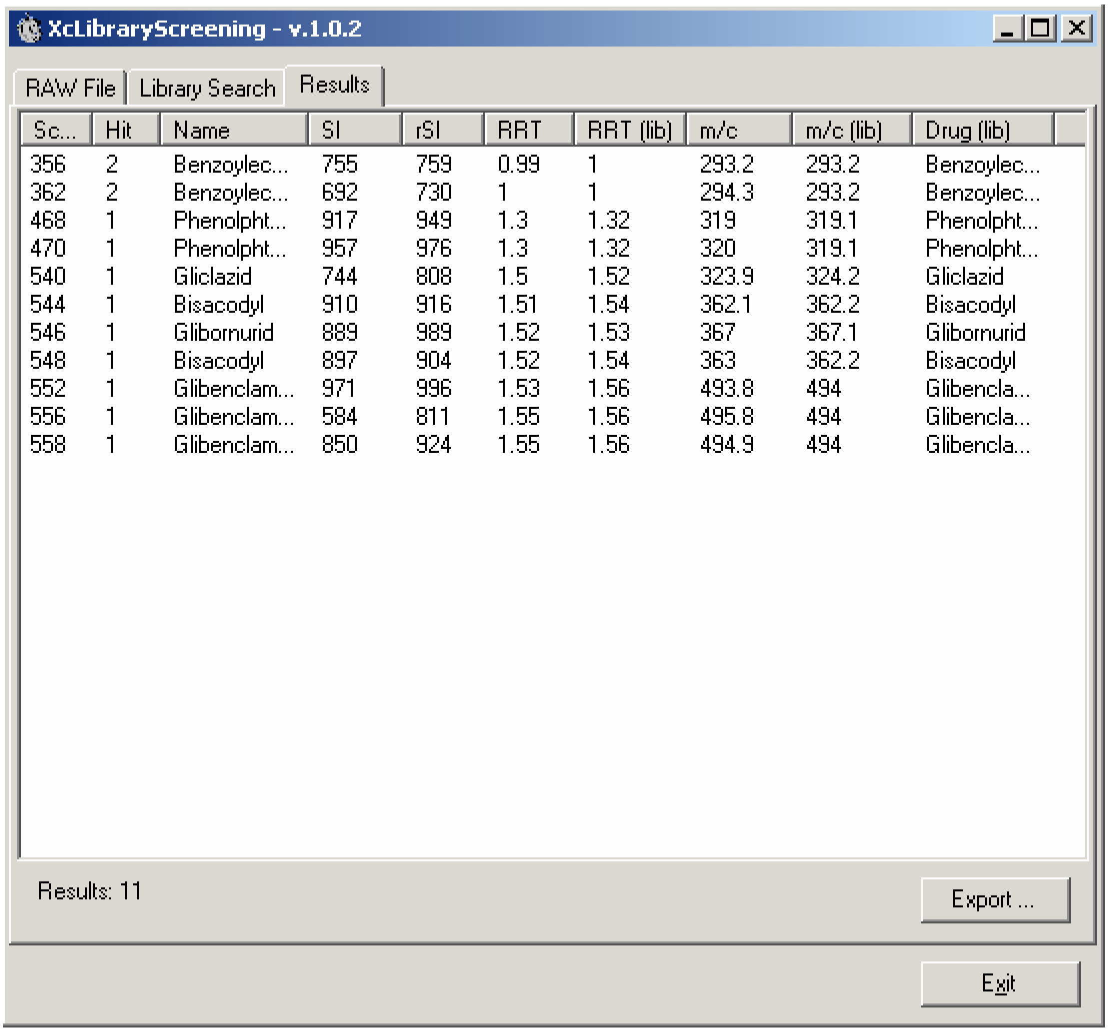
Direct Library Hits:

Scan	Hit	Name	SI	rSI	RRT	m/c
2	1	Minoxidi...	541	772	0.021	76.95
2	2	Risperid...	510	857	0.021	20.97
2	3	Oxymet...	390	397	0.021	1.1
2	4	Z-10-O...	373	376	0.021	0.6
2	5	Z-10-O...	311	311	0.021	0.1
2	6	E-10-O...	306	308	0.021	0.08
2	7	Desmet...	302	309	0.021	0.06
2	8	Metopro...	288	500	0.021	0.04
2	9	Nortrypti...	263	266	0.021	0.01
2	10	Heptab...	263	265	0.021	0.01
4	1	Nortrypti...	461	654	0.0585	64.12

Fig. 8. The library search parameters and the library can be specified. In this method the threshold of the match factors were set at 400, the RRT has to be within ± 5% and the maximum deviation of the mass-to-charge was two. It was allowed to return the ten best hits.

All 5 substances in a concentration of 1 µg/mL were identified with our procedure. Fig. 9 shows the output generated by the LC-MS-MS and the software. Gliclazide, bisacodyl, glibornuride and glibenclamide all have similar retention times. However, this was a minor problem because a refractory period was applied enabling to identify the compounds even though they were not chromatographically separated. Importantly, the co-eluting substances did not affect the MS-MS spectra in the presented procedure in contrast to single MS CID methods. Therefore, the analysis of unknown compound is more rapid using MS-MS.

Spectra obtained using MS with in source-CID are mostly identically as those produced by conventional MS-MS CID. But these MS spectra would be affected by co-eluting substances.



Sc...	Hit	Name	SI	rSI	RRT	RRT (lib)	m/c	m/c (lib)	Drug (lib)
356	2	Benzoylec...	755	759	0.99	1	293.2	293.2	Benzoylec...
362	2	Benzoylec...	692	730	1	1	294.3	293.2	Benzoylec...
468	1	Phenolpht...	917	949	1.3	1.32	319	319.1	Phenolpht...
470	1	Phenolpht...	957	976	1.3	1.32	320	319.1	Phenolpht...
540	1	Gliclazid	744	808	1.5	1.52	323.9	324.2	Gliclazid
544	1	Bisacodyl	910	916	1.51	1.54	362.1	362.2	Bisacodyl
546	1	Glibornurid	889	989	1.52	1.53	367	367.1	Glibornurid
548	1	Bisacodyl	897	904	1.52	1.54	363	362.2	Bisacodyl
552	1	Glibenclam...	971	996	1.53	1.56	493.8	494	Glibencla...
556	1	Glibenclam...	584	811	1.55	1.56	495.8	494	Glibencla...
558	1	Glibenclam...	850	924	1.55	1.56	494.9	494	Glibencla...

Results: 11

Fig. 9. In this result file the different hits are presented with their substance name, match factor, reverse match factor, RRT and mass-to-charge relation compared to the corresponding parameters in the library. The same substance can be found several times with different MS-MS spectra.

Serum samples spiked with decreasing concentrations of the tested drugs were analyzed in order to determine LODs. Each concentration from 0.005 to 4 µg/mL was extracted and analyzed two times. The LOD was set at the lowest concentrations where both samples fulfilled the mentioned requirements to identify a compound and hits were reported. The compounds were listed as hits by the small application program.

Out of 100 tested compounds, only 13 (amobarbital, acetylsalicylic acid, butalbital, carbromal, coumaphos, ibuprofen, methylphenidate, naproxen, pentobarbital, salicylic acid, secobarbital, spironolactone and thiopental) were not detectable with this LC-MS method. These compounds were identified neither at high therapeutic concentrations nor at low toxic concentrations with the described method. Most of the undetectable drugs were acidic compounds belonging to the class of analgesics or barbiturates. Generally these compounds have high serum concentrations and can easily be detected with HPLC-DAD.

Therapeutic and toxic serum concentration are listed in a review of Schulz et al (66). These data were taken as an orientation where the therapeutic concentration ended and the toxic concentration began. In general, therapeutic plasma concentration ranges or concentrations found after therapeutic dosing refer to trough levels at steady state. For a specific patient, it is often not possible to find the threshold between the therapeutic and toxic concentration. This is the case if tolerance develops and if drug interactions or additional diseases are involved.

Table VI shows the different LODs for 87 compounds detected either in the positive and/or in the negative mode. With our procedure all these compounds could be detected at high therapeutic drug concentration or at concentrations in the low toxic range. LOD was ≤ 100 ng/mL for 67% of the compounds. Most of drugs were better detected in the positive mode, especially compounds with chemical structures of amines such as neuroleptics, opioids and anti-depressants. In the negative mode a lower LOD was seen with molecules containing acidic sites like diclofenac.

For routine screening the combination of SPE, LC and APCI-MS represents an attractive alternative to the well-established technique of GC-MS. The SPE-APCI-MS screening method demonstrated to be suitable for routine measurements of serum samples. The method is highly specific because the substances are identified by their retention times, their molecular ions and characteristic fragments.

Rapid identification in screening experiments was achieved by the creation of the small application program. This program incorporates mass spectra data, molecular ions and retention time. With the presented method, the analysis of 1 serum sample can be performed in less than an hour.

Table VI. LODs in Serum Determined for 87 Drugs Applying Negative and Positive Ionization Mode.

	LOD (ng/mL)			LOD (ng/mL)	
	positive	negative		positive	negative
6-acetylmorphine	100		hydrochlorothiazide		4000
acebutolol	100		hydrocodone	50	
acenocoumarol		25	hydromorphone	50	
acetaminophen	500		imipramine	500	
acetazolamide		2000	indinavir	25	250
aloeemodine		50	levomepromazine	400	
alprazolam	50		lidocaine	250	
alprenolol	250		lopinavir	50	25
amiloride	100		lorazepam	100	100
amitryptiline	25		mefenamic acid	250	250
amprenavir	50	50	mepivacaine	100	
atenolol	25		metoprolol	250	
benzoylecgonine	50		morphine	50	
bisacodyl	100		nadolol	50	
brallobarbital		1000	nalbuphine	50	
bromadiolone	1000	250	nelfinavir	25	100
bupivacaine	250		nevirapine	50	
cannabidiol	100		norcodeine	100	
cannabinol	250		olanzapine	250	
canrenone	50		oxprenolol	250	
chlordiazepoxide	100		phenobarbital		2000
chlorophacinone		50	phenolphthalein	50	
chlorthalidone	1000		phenprocoumon	500	50
cinchocaine	25		pindolol	100	
citalopram	100		propranolol	100	
cocaethylene	500		propyphenazone	50	
cocaine	100		pseudoephedrine	100	
codeine	100		quetiapine	50	
coumachlor	500	50	rhein		50
coumatetralyl	1000	100	ritalinic acid	500	
crimidine	250		ritonavir	50	
delta-8-THC	100		saquinavir	10	25
delta-9-THC	100		sertraline	250	
diazepam	250		sotalol	25	
diclofenac	1000	500	thioridazine	500	
dihydrocodeine	25		timolol	100	
efavirenz		100	tolbutamide		250
emodine		50	torasemide	100	
ephedrine	500		trimipramine	250	
flupenthixol	100		tubocurarine	100	
furosemide		100	venlafaxine	250	
glibenclamide	25	50	warfarin	250	25
glibornuride	100	100	zuclopenthixol	100	
gliclazide	250	100			

5.2.5. A Semi-Quantitative Procedure with LC-MS

The chosen procedure enables to perform a semi-quantitative analysis. In a general unknown procedure it is not only important to determine the drugs potentially responsible for the toxic effect but also the approximate concentration of the compound. In the described procedure most of the compounds were detected at therapeutic concentrations. Some of the drugs like atenolol can even be identified at levels lower than the therapeutic concentration. Therefore, it is important to quantify the present drug in a GUS to have a first reference to the severity of the intoxication.

In the chosen program at least every second event is a full scan. A quantitative result of any compound can be calculated with the corresponding extracted ion chromatogram. Fig. 10 and 11 give an overview of all the extracted ion chromatograms of water sample extracted according the solid-phase extraction procedure.

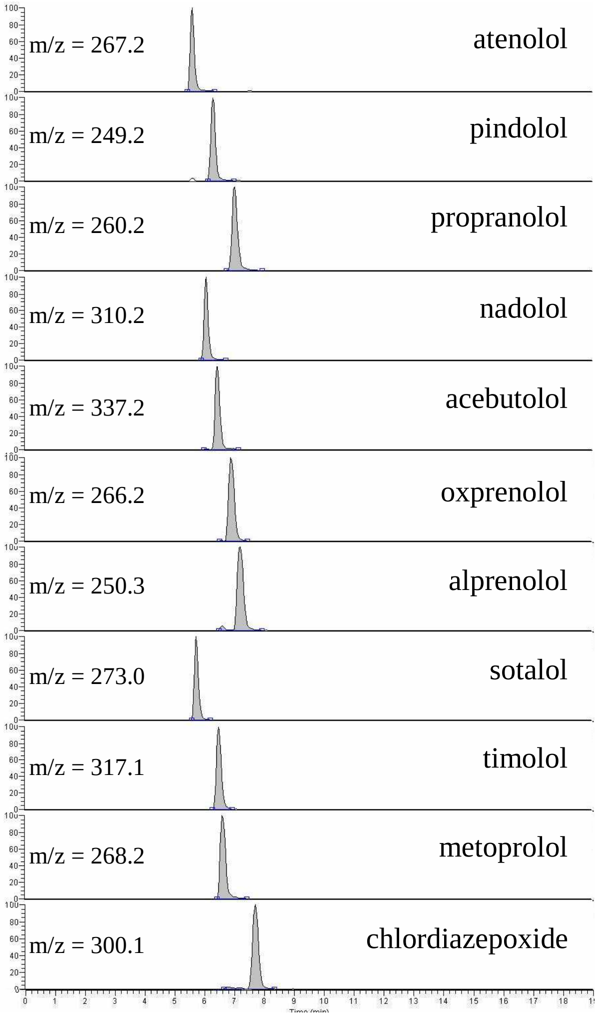


Fig. 10. XIC chromatograms of aqueous solutions spiked with compounds. 79 compounds are displayed in the positive mode.

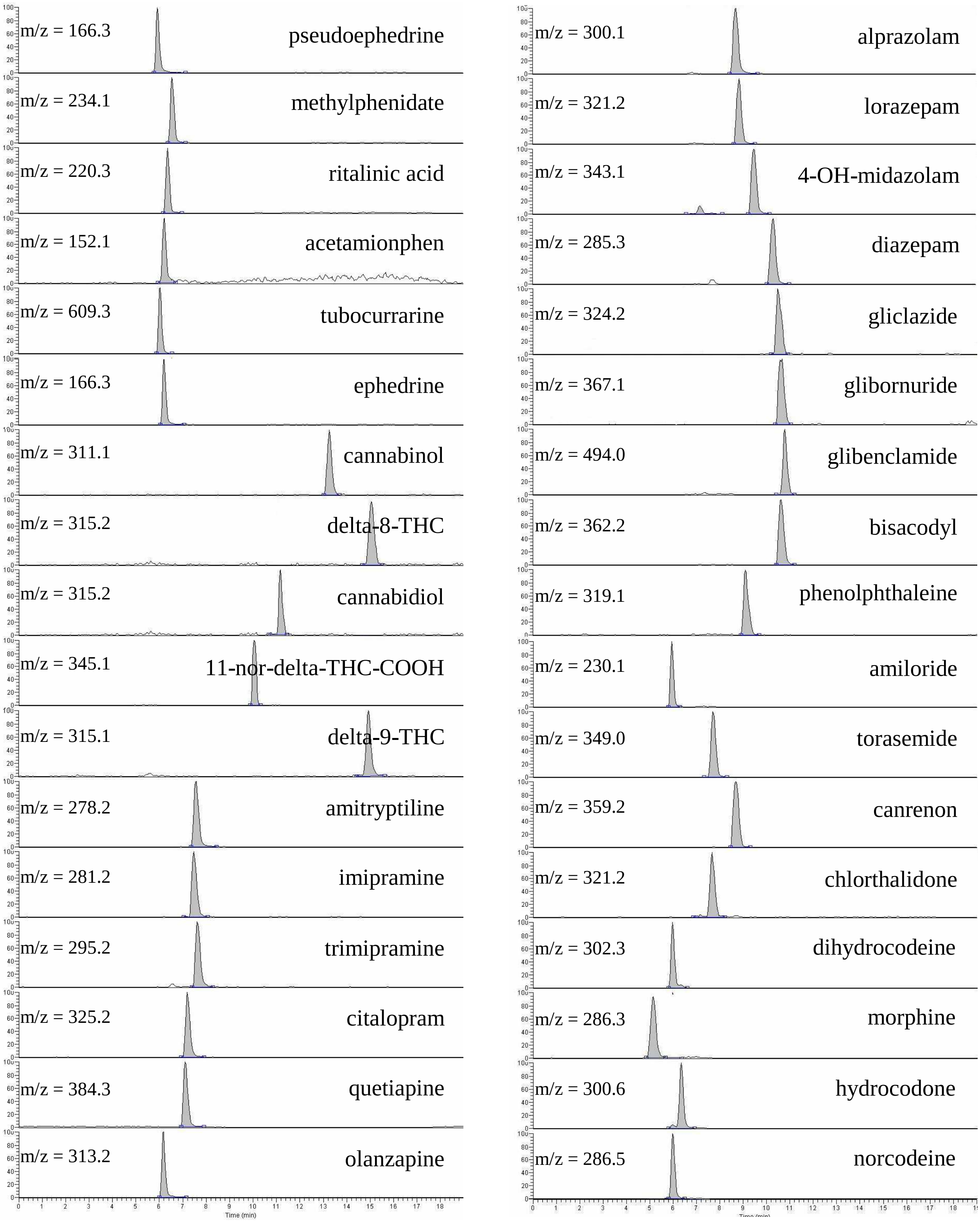


Fig. 10 (continued). XIC chromatograms of aqueous solutions spiked with compounds. 79 compounds are displayed in the positive mode.

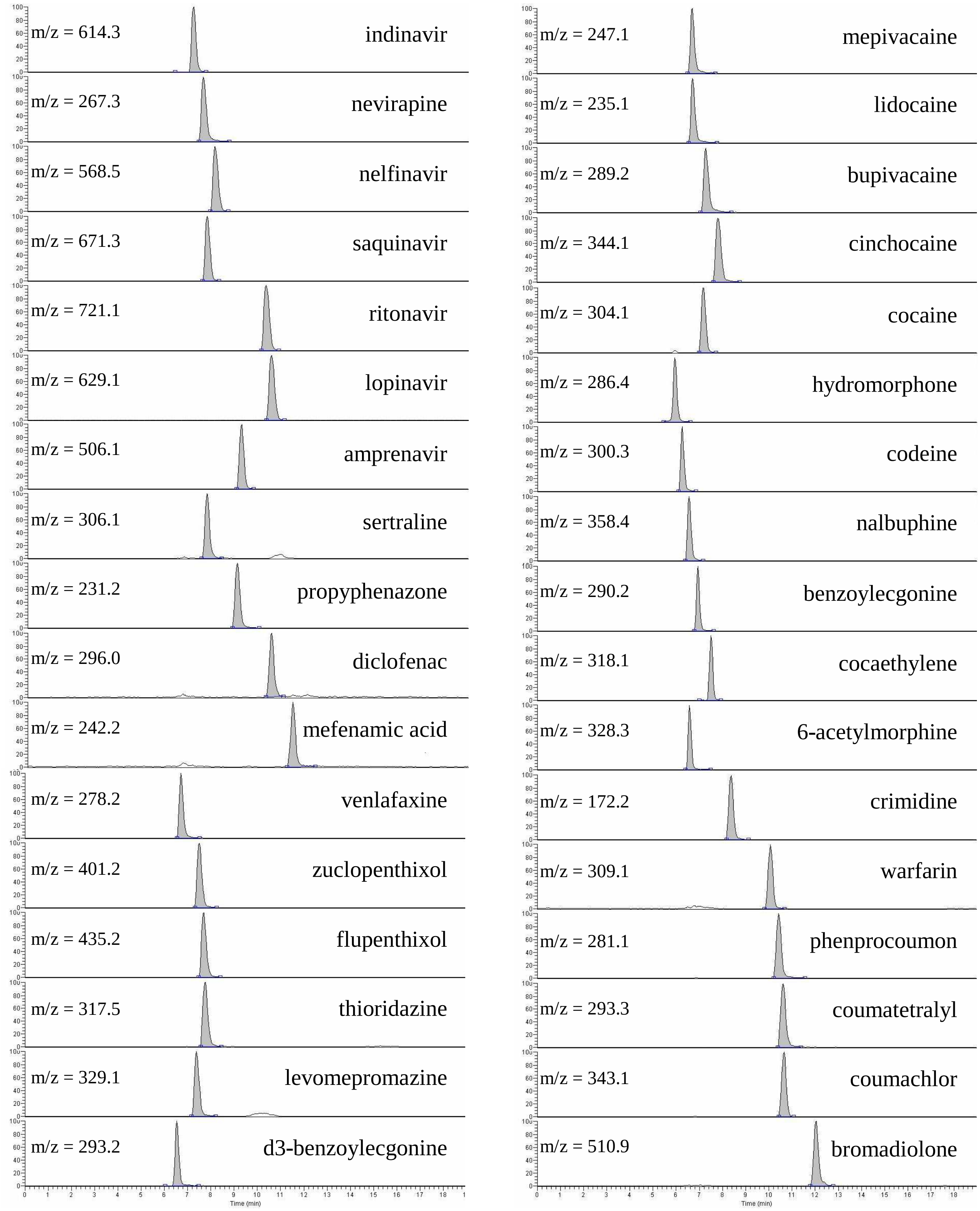


Fig. 10 (continued). XIC chromatograms of aqueous solutions spiked with compounds. 79 compounds are displayed in the positive mode.

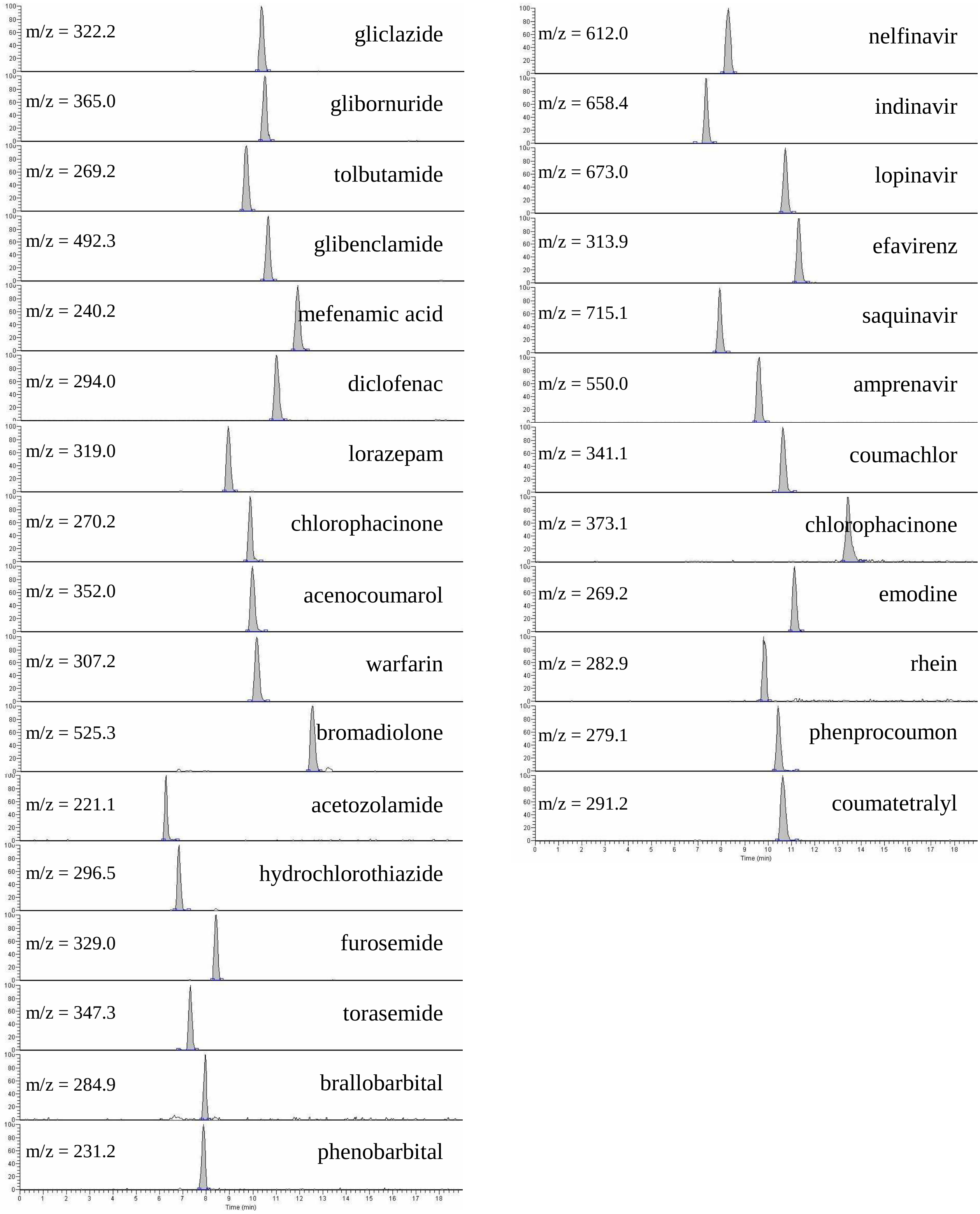


Fig. 11. XIC chromatograms of aqueous solutions spiked with compounds in the negative mode. 29 compounds are displayed in the negative mode.

Fig. 10 shows the extracted ion chromatograms of 79 compounds detected in the positive mode, Fig. 11 shows the chromatograms of 29 compounds detected in the negative mode. Peak areas of the compounds spiked in serum (at a concentration of 1 μg/mL) were recorded and the area ratios (peak area of the compound relative to the peak area of the IS) were attached to the library together with the RRTs and the corresponding molecular ions (Table VII). Concentration of unknown compounds can be determined with the area ratio.

$$\text{Concentration}_{\text{unknown compound}}\ (\mu\text{g/mL}) = \text{Area}_{\text{unknown compound}} / (\text{Area Ratio} * \text{Area}_{\text{IS}})$$

Further studies have to be done to approve the performance of the quantitative analysis. The ionization process can be more affected for certain substances than for others. In this study only one internal standard was applied, therefore a precise quantification is not possible. But preliminary semi-quantitative experiments are promising. This fits perfectly into the picture of screening procedures.

Most of the GUS described yet provide only a qualitative result (44, 48, 53). A specific quantitative procedure has to be performed in addition.

Table VII. Relative Retention Times (RRT), Molecular Ions, and Area Ratios (Area Compound / Area Internal Standard) of the Screened Compounds.

	Compound	RRT	Molecular Ion	Area Ratio
1	10,11-dihydro-10-OH-carbamazepine	1.11	255.0	0.89
2	11-nor-delta-THC-COOH	1.86	345.1	0.80
3	11-OH-delta-9-THC	1.87	331.0	n. d.
4	17-methylmorphinane	1.09	258.4	0.28
5	2 C-B	1.06	259.9	0.25
6	2 C-H	0.98	181.9	n. d.
7	2-OH-desipramine	1.07	283.3	0.41
8	3-OH-bromazepam	1.15	334.0	n. d.
9	3-OH-flunitrazepam	1.32	330.0	0.28
10	4-OH-clobazam	1.25	317.1	0.08
11	4-OH-clobazam adduct	1.25	333.8	0.12
12	4-OH-midazolam	1.44	343.1	4.10
13	6-acetylcodeine	1.03	342.2	n. d.
14	6-acetylmorphine	0.94	328.2	2.36
15	7-acetaminoclonazepam	1.11	328.1	2.55
16	7-aminoclonazepam	1.12	286.2	3.13
17	7-aminoflunitrazepam	1.17	284.2	n. d.
18	7-aminonitrazepam	1.00	252.2	4.05
19	8-OH-amoxapine	1.05	330.0	0.69
20	9-OH-risperidone	1.04	427.2	3.15
21	acebutolol	1.00	337.2	2.70
22	acenocoumarol neg	1.51	352.0	0.38
23	acetozolamide neg	0.99	221.1	0.02
24	alimemazine	1.18	299.1	1.17
25	aloeemodine neg	1.50	270.2	0.29
26	alpha-OH-alprazolam	1.25	325.2	n. d.
27	alpha-OH-midazolam	1.23	342.1	3.23
28	alprazolam	1.32	309.5	8.92
29	alprenolol	1.10	250.3	0.82
30	amiloride neg	0.87	211.4	0.00
31	amiloride	0.88	230.1	0.92
32	aminophenazone	0.94	232.0	1.92
33	amiodarone	1.51	646.0	2.56
34	amisulpiride	0.98	370.1	10.55
35	amitryptiline	1.17	278.2	4.48
36	amoxapine	1.12	314.2	2.77
37	amphetamine	0.96	135.9	n. d.
38	ampicilline neg	0.93	348.4	0.05
39	amprenavir	1.42	506.1	7.70
40	amprenavir adduct neg	1.42	550.0	0.25
41	apomorphine	0.97	268.2	0.24
42	aripiprazol	1.17	448.3	4.86
43	atenolol	0.87	267.2	4.32
44	atropine	0.99	290.2	n. d.
45	azacyclonol	1.07	268.0	n. d.
46	azathioprine	0.99	278.1	n. d.
47	azosemide neg	1.39	369.4	0.16
48	BDB	1.00	193.9	n. d.

Table VII (continued). Relative Retention Times (RRT), Molecular Ions, and Area Ratios (Area Compound / Area Internal Standard) of the Screened Compounds.

	Compound	RRT	Molecular Ion	Area Ratio
49	bendroflumethiazide neg	1.38	420.1	0.50
50	benzoylecgonine	1.00	290.2	2.64
51	benzoylecgonine-d3	1.00	293.2	2.00
52	benzthiazide adduct	1.33	448.8	0.09
53	benzthiazide neg	1.34	430.2	0.45
54	betamethasone	1.69	393.2	0.19
55	betamethasone adduct	1.68	505.1	3.48
56	biperidene	1.17	312.2	1.75
57	bisacodyl	1.54	362.2	0.60
58	brallobarbital neg	1.24	284.9	0.02
59	bromadiolone	1.86	510.9	1.02
60	bromadiolone neg	1.86	525.3	0.14
61	bromazepam	1.25	318.3	n. d.
62	brotizolam	1.38	395.0	5.14
63	buclizine	1.51	433.2	n. d.
64	buformine	0.69	158.3	0.02
65	bumetanide	1.42	365.0	0.27
66	bumetanide neg	1.43	363.2	0.13
67	bumetanide adduct neg	1.43	408.9	0.36
68	bupivacaine	1.08	289.2	3.00
69	buprenorphine	1.13	450.3	4.03
70	butalbital neg	1.28	223.4	0.02
71	camazepam	1.57	371.7	n. d.
72	cannabidiol	2.07	315.1	0.22
73	cannabinol	2.46	311.1	0.70
74	canrenone acid	1.28	359.2	0.68
75	captopril neg	1.08	215.9	0.01
76	carbamazepine	1.29	237.1	6.33
77	carbamazepine-10-11-epoxide	1.19	253.0	1.85
78	carisoprodol	1.36	261.0	n. d.
79	chlorcyclizine	1.16	300.9	n. d.
80	chlordiazepoxide	1.18	300.1	3.28
81	chlorophacinone neg	2.00	373.1	0.31
82	chloroquine	0.92	320.3	3.56
83	chlorpheniramine	1.06	275.0	n. d.
84	chlorpromazine	1.20	319.1	1.27
85	chlorprothixene	1.23	316.1	3.68
86	chlorthalidone	1.13	321.2	0.24
87	cinchocaine	1.15	344.1	11.48
88	citalopram	1.11	325.2	2.68
89	clobazam	1.45	301.0	0.13
90	clomethiazole	1.35	162.1	0.40
91	clomipramine	1.21	315.1	n. d.
92	clonazepam	1.37	316.2	2.90
93	clonidine	0.94	230.2	n. d.
94	clopenthixol	1.22	401.2	1.50
95	clorazepate	1.43	271.1	2.53
96	clotiapine	1.17	344.1	n. d.

Table VII (continued). Relative Retention Times (RRT), Molecular Ions, and Area Ratios (Area Compound / Area Internal Standard) of the Screened Compounds.

	Compound	RRT	Molecular Ion	Area Ratio
97	clovoxamine	1.14	285.0	1.08
98	cloxazolam	1.01	349.1	0.70
99	clozapine	1.11	327.1	3.93
100	clozapine-N-oxide	1.11	343.0	n. d.
101	cocaethylene	1.07	318.2	1.86
102	cocaine	1.06	304.2	2.26
103	codeine	0.93	300.3	3.98
104	codeine-6-glucuronide	0.90	476.2	n. d.
105	coumachlor	1.62	343.1	1.96
106	coumachlor neg	1.62	341.1	0.61
107	coumatetralyl	1.63	293.3	1.24
108	coumatetralyl neg	1.63	291.2	0.40
109	crimidine	1.29	172.2	5.08
110	cyclizine	1.11	267.0	n. d.
111	cyclopenthiazide neg	1.40	378.2	0.31
112	cyclothiazide	1.37	390.0	n. d.
113	cyclothiazide neg	1.36	388.3	n. d.
114	delorazepam	1.45	305.2	2.52
115	delorazepam neg	1.44	267.2	0.22
116	delta-8-THC	2.78	315.1	0.32
117	delta-9-THC	2.71	315.1	1.86
118	delta-9-THC d3	2.70	318.3	1.86
119	demethylcitalopram	1.12	311.1	2.58
120	demoxepam	1.25	287.1	n. d.
121	desalkylflurazepam	1.40	289.2	3.32
122	desalkylremifentanil	1.01	363.0	1.51
123	desipramine	1.15	267.1	n. d.
124	desmethylchlorodiazepoxide	1.12	285.9	0.26
125	desmethylchlorprothixene	1.19	302.1	n. d.
126	desmethylclobazam	1.35	287.1	0.02
127	desmethylclobazam adduct	1.35	303.7	n. d.
128	desmethylclomipramine	1.22	301.1	1.21
129	desmethylclozapine	1.08	313.4	n. d.
130	desmethyldiazepam	1.43	271.1	n. d.
131	desmethyldoxepine	1.12	266.1	n. d.
132	desmethylflunitrazepam	1.33	300.2	2.86
133	desmethyllevomepromazine	1.15	301.1	n. d.
134	desmethyllevomepromazine sulfoxide	1.04	317.3	n. d.
135	desmethylmaprotiline	1.16	264.1	2.58
136	desmethylmelitracene	1.21	278.1	3.88
137	desmethylmianserine	1.11	251.1	n. d.
138	desmethylmirtazepine	1.01	252.2	1.56
139	desmethylnortryptiline	1.15	250.3	n. d.
140	desmethylsertraline	1.18	292.1	n. d.
141	desmethyltrimipramine	1.18	281.1	1.81
142	desmethylvenlafaxine	0.98	264.1	3.25
143	dextrometorphan	1.12	272.2	2.52
144	diazepam	1.57	285.3	6.26

Table VII (continued). Relative Retention Times (RRT), Molecular Ions, and Area Ratios (Area Compound / Area Internal Standard) of the Screened Compounds.

	Compound	RRT	Molecular Ion	Area Ratio
145	diazepambenzophenon	2.03	246.1	1.09
146	dibenzepine	1.05	296.0	6.67
147	diclofenac	1.61	296.0	0.82
148	diclofenac neg	1.61	294.0	0.18
149	didemethylcitalopram	1.11	297.0	1.50
150	didemethyltrimipramin	1.15	267.2	1.62
151	dihydrocodein	0.92	302.3	5.78
152	dihydrocodeine-6-beta-D-glucuronide	0.89	478.2	n. d.
153	dihydromorphine	0.62	288.2	2.16
154	dimethoxyphenethylamine	0.92	181.9	n. d.
155	dimetindene	1.10	293.1	3.14
156	diphenhyrdramine	1.12	255.9	1.56
157	diphenoxylate	1.32	453.3	n. d.
158	diphylline	0.91	255.1	n. d.
159	dothiepine	1.16	296.1	4.05
160	doxepine	1.12	280.4	n. d.
161	doxycycline	1.07	446.0	0.58
162	E-10-OH-amitryptiline	1.05	294.1	2.18
163	E-10-OH-nortryptiline	1.05	280.1	1.03
164	EDDP	1.15	278.3	3.91
165	efavirenz neg	1.68	314.3	0.36
166	emodine neg	1.69	269.5	0.56
167	enalapril	1.11	377.2	2.07
168	enalapril neg	1.11	375.5	0.02
169	ergotamin	1.10	582.0	1.80
170	ergotamine neg	1.09	580.2	0.06
171	estazolam	1.31	295.2	5.19
172	ethacrinic acid neg	1.52	301.2	0.17
173	ethambutol	0.92	205.1	4.20
174	ethylmorphine	0.96	314.2	n. d.
175	fentanyl	1.12	337.5	5.27
176	flunitrazepam	1.42	314.1	3.47
177	fluorescein	1.33	333.3	n. d.
178	fluorescein neg	1.32	331.3	n. d.
179	fluoxetine	1.18	309.9	n. d.
180	flupenthixol	1.17	435.2	3.94
181	fluphenazine	1.22	438.2	n. d.
182	flurazepam	1.11	388.1	5.13
183	fluvoxamine	1.16	319.0	1.54
184	furosemide neg	1.30	329.0	0.25
185	glafenine	1.00	373.1	3.16
186	glafenine neg	1.00	371.0	0.06
187	glafenic acid	1.05	299.1	0.88
188	glafenic acid neg	1.06	297.0	0.03
189	glibenclamid neg	1.56	492.3	0.55
190	glibenclamide	1.56	494.0	0.30
191	glibornuride	1.53	367.1	0.14
192	glibornuride neg	1.53	365.0	0.14

Table VII (continued). Relative Retention Times (RRT), Molecular Ions, and Area Ratios (Area Compound / Area Internal Standard) of the Screened Compounds.

	Compound	RRT	Molecular Ion	Area Ratio
193	gliclazide	1.52	324.2	0.12
194	gliclazide neg	1.52	322.2	0.08
195	haloperidol	1.15	376.1	3.53
196	heptabarbital	1.32	251.0	0.32
197	heroine	1.02	370.3	n. d.
198	hexobarbital	1.35	236.9	0.01
199	HMA	0.88	181.9	n. d.
200	HMMA	0.89	196.2	n. d.
201	hordenine	0.63	166.0	0.12
202	hydrochlorothiazide neg	1.08	296.5	0.01
203	hydrocodone	0.98	300.6	5.46
204	hydrocortisone adduct neg	1.20	407.1	0.19
205	hydroflumethiazide neg	1.15	330.3	0.08
206	hydroflumethiazide adduct neg	1.15	375.9	0.06
207	hydromorphine	0.88	286.2	n. d.
208	hydromorphone	0.88	286.4	2.88
209	hydroxy imipramine	1.08	297.2	0.62
210	hydroxy levomepromazine	1.10	345.2	0.45
211	hydroxyphenamate	1.20	191.9	n. d.
212	imipramine	1.16	281.2	1.62
213	indinavir	1.09	614.3	13.94
214	indinavir adduct neg	1.09	658.4	0.75
215	isocarboxazide	1.24	232.1	n. d.
216	isoniazide	0.58	138.1	0.05
217	ketamine	0.99	238.0	3.79
218	labetolol	1.06	329.0	n. d.
219	lamotrigine	1.02	256.2	0.33
220	l-ephedrine	0.93	166.3	2.38
221	levallorphan	1.04	284.2	n. d.
222	levomepromazine	1.12	329.1	1.28
223	levomepromazine sulfoxide	1.06	345.2	2.35
224	levorphanol	1.00	258.4	0.40
225	lidocaine	1.00	235.1	2.40
226	lobeline	1.14	338.2	n. d.
227	lofepramin	1.38	419.1	2.06
228	lopinavir	1.58	629.1	6.08
229	lopinavir adduct neg	1.58	673.0	1.66
230	lorazepam neg	1.35	319.0	0.15
231	lorazepam	1.35	321.2	3.00
232	lormetazepam	1.47	335.0	1.53
233	lormetazepam neg	1.46	298.0	0.25
234	LSD	1.07	324.3	2.34
235	maprotiline	1.17	278.1	3.83
236	MBDB	1.00	208.0	n. d.
237	MDA	0.97	179.9	n. d.
238	MDEA	1.00	207.9	5.92
239	MDMA	0.99	194.0	n. d.
240	mebeverine	1.16	430.2	2.48

Table VII (continued). Relative Retention Times (RRT), Molecular Ions, and Area Ratios (Area Compound / Area Internal Standard) of the Screened Compounds.

	Compound	RRT	Molecular Ion	Area Ratio
241	meclizine	1.35	391.1	n. d.
242	medazepam	1.18	271.2	n. d.
243	mefenamic acid neg	1.74	240.2	6.10
244	mefenamic acid	1.74	242.2	n. d.
245	melitracene	1.22	292.2	1.39
246	mephenoxalone adduct	1.17	240.9	1.37
247	mephentermin	0.99	164.0	n. d.
248	mepivacaine	0.99	247.1	2.34
249	meprobamate	1.15	218.9	n. d.
250	mescaline	0.94	211.9	8.24
251	mesoridazine	1.09	387.1	3.26
252	metalazone	1.29	366.0	2.60
253	metalazone neg	1.28	364.5	n. d.
254	metamphetamine	0.98	150.0	n. d.
255	methadone	1.21	310.3	n. d.
256	methapyrilene	1.06	262.0	n. d.
257	methaqualone	1.43	251.2	n. d.
258	methocarbamol	1.11	242.0	5.84
259	methohexital	1.50	263.0	n. d.
260	metixene	1.20	310.4	3.04
261	metoclopramide	1.00	300.1	0.04
262	metoprolol	1.01	268.2	0.01
263	metronidazolum	0.98	172.1	3.34
264	mianserine	1.11	265.2	0.14
265	mianserine-N-oxide	1.14	281.1	4.54
266	midazolam	1.15	326.2	0.70
267	minoxidil	0.99	210.1	6.04
268	mirtazapine	1.01	266.1	1.23
269	moclobemide	1.00	269.1	16.86
270	morphine	0.78	286.2	3.29
271	morphine-6-glucuronide	0.82	462.1	n. d.
272	MPPH	1.36	267.1	0.02
273	N1-OH-ethylflurazepam	1.35	333.1	1.72
274	nadolol	0.94	310.2	4.86
275	nalbuphine	0.97	358.3	n. d.
276	nalorphine	0.91	312.2	2.50
277	naloxone	0.92	328.1	12.00
278	naltrexone	0.94	342.1	n. d.
279	naltrexone neg	0.93	341.5	4.32
280	N-desmethylclozapine	1.08	313.4	5.62
281	nelfinavir	1.23	568.5	0.04
282	nelfinavir adduct neg	1.23	612.2	n. d.
283	N-ethyloxazepam	1.52	315.0	n. d.
284	nevirapine	1.16	267.3	0.08
285	nifedipine adduct neg	1.47	391.3	0.26
286	nifedipine neg	1.47	345.1	2.95
287	nifedipin metabolite	1.47	329.0	1.30
288	nitrazepam	1.35	282.1	10.51

Table VII (continued). Relative Retention Times (RRT), Molecular Ions, and Area Ratios (Area Compound / Area Internal Standard) of the Screened Compounds.

	Compound	RRT	Molecular Ion	Area Ratio
289	nomifensine	1.06	239.1	2.44
290	norcodeine	0.92	286.3	0.15
291	norfentanyl	0.99	233.0	0.05
292	normorphine	0.77	272.1	2.22
293	nortryptiline	1.17	362.3	0.33
294	noscapine	1.10	414.1	2.56
295	noxiptiline	1.16	295.0	5.27
296	nylindrine	1.07	300.1	5.81
297	ofloxacine	0.96	362.3	0.09
298	olanzapine	0.93	313.2	3.80
299	opipramol	1.11	364.2	1.24
300	oxazepam	1.33	287.1	7.20
301	oxcarbazepine	1.22	253.0	1.78
302	oxprenolol	1.06	266.2	0.72
303	oxycodone	0.96	316.1	n. d.
304	oxymetazoline	1.10	261.2	2.84
305	oxymorphone	0.82	302.1	n. d.
306	papaverine	1.06	340.5	4.18
307	paracetamol	0.95	152.1	2.12
308	paroxetine	1.14	330.1	8.34
309	PCP	1.10	243.9	4.10
310	penfluridol	1.35	524.7	2.84
311	pentazocine	1.07	286.1	0.57
312	perazine	1.18	340.2	n. d.
313	periciazine	1.12	366.1	3.31
314	perphenazine	1.18	404.3	6.26
315	pethidine	1.05	248.1	1.16
316	phenacetin	1.23	180.2	2.66
317	phenazopyridine	1.21	214.2	0.89
318	phendimetrazine	0.96	192.1	0.80
319	phenindamine	1.13	262.1	n. d.
320	pheniramine	0.99	241.0	0.28
321	phenobarbital neg	1.23	231.2	0.25
322	phenolphthalein	1.32	319.1	n. d.
323	phenprocoumon	1.60	281.1	0.41
324	phenprocoumon neg	1.60	279.1	n. d.
325	phenylbutazone neg	1.68	307.5	0.28
326	pholcodine	0.60	399.2	0.08
327	pimozide	1.22	462.2	0.12
328	pinazepam	1.62	309.3	4.10
329	pindolol	0.98	249.2	n. d.
330	PMA	0.97	165.9	2.36
331	PMMA	0.99	179.9	2.55
332	prazepam	1.75	325.1	3.13
333	procaine	0.92	237.0	n. d.
334	promazine	1.16	285.0	4.05
335	promethazine	1.15	285.0	0.69
336	propoxyphene	1.18	339.9	3.15

Table VII (continued). Relative Retention Times (RRT), Molecular Ions, and Area Ratios (Area Compound / Area Internal Standard) of the Screened Compounds.

	Compound	RRT	Molecular Ion	Area Ratio
337	propranolol	1.09	260.2	2.70
338	propylhexedrine	1.05	156.1	0.38
339	propyphenazone	1.40	231.2	0.02
340	protryptiline	1.15	264.1	1.17
341	pseudoephedrine	0.93	166.3	0.29
342	psilocyn	0.91	205.0	n. d.
343	pyrilamine	1.08	286.0	3.23
344	pyrrobutamine	1.20	312.1	8.92
345	quazepam	1.79	387.1	0.82
346	quazepam neg	1.79	384.9	0.00
347	quetiapine	1.10	384.3	0.92
348	quinine	0.99	325.2	1.92
349	ranitidine	0.88	315.1	2.56
350	ranitidine adduct neg	0.88	358.7	10.55
351	ranitidine neg	0.88	313.1	4.48
352	remifentanil	1.04	377.2	2.77
353	rhein neg	1.49	283.1	n. d.
354	risperidone	1.05	411.0	0.05
355	ritalinic acid	0.98	220.3	7.70
356	ritonavir	1.54	721.1	0.25
357	ropivacaine	1.06	275.1	0.24
358	saquinavir	1.18	671.3	4.86
359	saquinavir adduct neg	1.18	715.1	4.32
360	scopolamine	0.96	304.1	n. d.
361	secobarbital neg	1.37	237.1	n. d.
362	sertraline	1.19	306.1	n. d.
363	sotalol	0.90	273.0	0.16
364	strychnine	0.97	335.3	n. d.
365	sulforidazine	1.12	403.2	0.50
366	sulpiride	0.90	342.2	2.64
367	tadalafil	1.34	390.1	2.00
368	temazepam	1.45	301.0	0.09
369	tetrazepam	1.72	289.2	0.45
370	thalidomide neg	1.19	258.3	0.19
371	thebaine	1.04	312.4	3.48
372	thenyldiamine	1.06	262.0	1.75
373	theophylline neg	0.93	179.2	0.60
374	thiabutizide neg	1.33	352.4	0.02
375	thioproperazine	1.16	447.2	1.02
376	thioridazine	1.18	371.5	0.14
377	tiapride	0.93	329.1	n. d.
378	timolol	1.00	317.1	5.14
379	tolbutamide neg	1.42	269.2	n. d.
380	torasemide neg	1.14	347.3	0.02
381	torasemide	1.14	349.0	0.27
382	tramadol	1.02	264.0	0.13
383	trazodone	1.08	372.2	0.36
384	triamterene	0.97	254.3	3.00

Table VII (continued). Relative Retention Times (RRT), Molecular Ions, and Area Ratios (Area Compound / Area Internal Standard) of the Screened Compounds.

	Compound	RRT	Molecular Ion	Area Ratio
385	triazolam	1.34	343.2	4.03
386	trichlormethiazide neg	1.25	379.7	0.02
387	trifluperidol	1.17	410.1	n. d.
388	triflupromazine	1.24	353.1	0.22
389	trimethoprim	0.96	291.1	0.70
390	trimipramine	1.18	295.2	0.68
391	trimipramine metabolite 1	1.20	250.3	0.01
392	tripelenamine	1.07	256.0	6.33
393	tubocurarine	0.94	609.3	1.85
394	tybamate	1.44	274.9	n. d.
395	vardenafil	1.07	489.3	n. d.
396	venlaflaxine	1.02	278.2	3.28
397	verapamil	1.16	455.3	0.31
398	voriconazole	1.36	350.0	3.56
399	warfarin	1.53	309.1	n. d.
400	warfarin neg	1.53	307.2	1.27
401	xylometazoline	1.15	245.2	3.68
402	Z-10-OH-amitryptiline	1.09	294.1	0.24
403	zolpidem	1.06	308.2	11.48
404	zopiclone	1.00	389.0	2.68
405	zuclopenthixol	1.14	401.2	0.13

5.2.6. Comparison of LC-MS and HPLC-DAD

The LC-MS GUS procedure in the positive mode was compared with the same procedure in the negative mode and the HPLC-DAD analysis for 100 substances. On-line solid-phase extraction procedure on Hysphere GP resin cartridges was used for LC-MS and off-line SPE on Oasis HLB cartridges was used for HPLC-DAD.

It was found that LC-MS in the positive mode was able to detect 73 out of 100 compounds, versus 28% for LC-MS in the negative mode and 61% for HPLC-DAD. 29% of all the compounds were only detected by LC-MS in the positive mode, versus 6% in the negative mode and 13% for HPLC-DAD (Fig. 12). These results showed the efficiency of LC-MS in both modes in combination with HPLC-DAD as a screening procedure in toxicology.

MS-MS in toxicology brings higher specificity and selectivity and more structural information to explore an unknown chromatographic peak. For the most drugs or toxic compounds, the LOD was lower with the LC-MS than with the HPLC-DAD procedure.

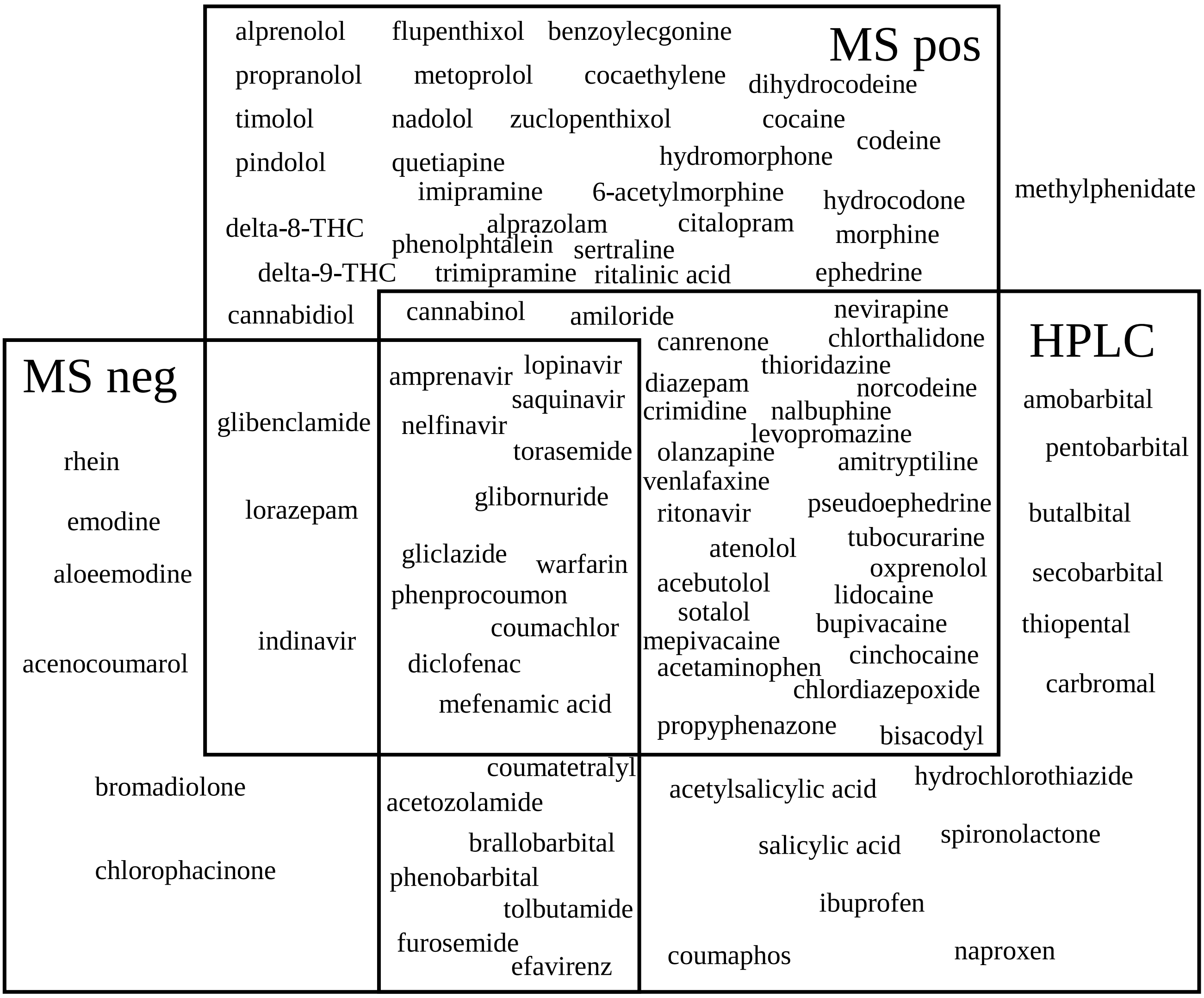


Fig. 12. Comparison of LC-MS in a positive mode, LC-MS in a negative mode and HPLC-DAD general unknown screening procedure for the detection of 100 substances at a low toxic concentration.

5.3. CASE REPORTS

The DDA LC-MS-MS system has been applied to several cases. The following examples illustrate the application of the system for clinical investigations. The results of urine and serum samples analysis using SPE-LC-MS-MS were compared to the results obtained with a conventional GUS technique (including immunoassay, REMEDi and LC-MS). REMEDi (Biorad) is an HPLC-based broad-spectrum drug profiling system. It is used to detect and identify drugs in clinical serum and urine samples. The REMEDi is designed to identify basic and neutral drugs and their metabolites. The system can also be used for the identification of benzodiazepines. These systems were applied in the present laboratory for several years.

Seventeen urine and twentyfive serum samples of potentially intoxicated individuals and drug addicts were analyzed (Table VIII, IX).
Urine samples were treated like serum samples and were analyzed with both systems. The LC-MS-MS system found in addition to the REMEDi 7-aminoflunitrazepam (case 5), mirtazapine (case 8), zolpidem (case 8 and 10), metoprolol (case 8), phenprocoumon (case 10), ephedrine, mephentermine, lidocaine (all in case 11), torasemide, propylhexedrine (both in case 12) and 3-OH-bromazepam (case 14). The LC-MS-MS procedure failed to detect diphenhydramine, the metabolite of diphenhydramine (both in case 5), heroin, 6-acetylmorphine (case 5 and 10), hydrocodone (case 10), phenobarbital (case 12), a metabolite of chlorprothixene (case 13), quinine (case 14), 7-OH-quetapine and N-OH-ethylflurazepam (case 16).

A number of drugs in serum have been detected by the DDA approach, which were not found by the REMEDi system including metoclopramide (case 19), amisulpiride, atenolol (both case 25), mefenamic acid (case 27 and 37), codeine-6-glucuronide (38) and lamotrigine (case 27). On the other hand atracurium (case 19), dipyrone (case 19 and 34), citalopram (case 25 and 31), venlafaxine and its metabolite, tramadol (both case 27), fluconazole, bisoprolol (both case 40) and acetaminophen (case 19 and 38) were missed by the new developed system.

Table VIII. Comparison of DDA LC-MS-MS Procedure to Conventional GUS Technique (Immunoassay and REMEDi) of Urine Samples.

No.	Immunoassay	REMEDi	DDA LC-MS-MS	Comment/LC-MS Confirmation
1			Lidocaine	Only Benzodiazepine REMEDi
	Cocaine		Cocaine, Benzoylecgonine	Cocaine, Benzoylecgonine
	Methadone, EDDP		Methadone, EDDP	
	Benzodiazepine	Temazepam, Desmethyldiazepam	Oxazepam, Oxazepam-glucuronide, Temazepam, Temazepamglucuronide, Desmethyldiazepam	
	Cannabis			
2	Opiate	Normorphine, Morphine, Codeine-6-glucuronide, Norcodeine	Normorphine, Morphine, Codeine-6-glucuronide, Codeine, Norcodeine	Morphine, Morphine-glucuronides, Codeine-6-glucuronide, Codeine, Norcodeine
3	Opiate	Morphine, Codein-6-glucuronide, Codeine	Normorphine, Codeine-6-glucuronide, Morphine-glucuronide, Codeine	
		Atenolol	Atenolol	
4	Cocaine	Cocaine, Benzoylecgonine	Cocaine, Benzoylecgonine, Cocaethylene	Alcohol
		Mepivacaine	Mepivacaine	
5	Opiate, 6-Acetylmorphine	Morphine, Morphine-glucuronide	Morphine, Morphineglucuronide	Morphine, Morphine-glucuronide, Heroin, 6-Acetylmorphine
		Trimipramine	Hydroxyimipramine, Trimipramine	No immunoassay
	Benzodiazepine	Desmethyldiazepam, Temazepam, Oxazepam,	Desmethyldiazepam, Temazepam, Temazepamglucuronide, Oxazepamglucuronide, 7-Aminoflunitrazepam,	
		Diphenhydramine, Metabolite of Diphenhydramine,		
	Methaqualone		Methaqualone	
	Cannabis			
6	Methadone, EDDP	Methadone, EDDP	EDDP	
	Opiate	Hydrocodone, Dihydromorphine, Hydromorphone,	Hydrocodone, Dihydrocodeine, Dihydrocodeine-6-glucuronide, Norcodeine,	Hydrocodone, Dihydrocodeine, Morphine-glucuronide
	Benzodiazepine	Desmethyldiazepam, Temazepam	Desmethyldiazepam, Temazepamglucuronide, Oxazepam, Oxazepam-glucuronide	
	Cannabis		11-nor-delta-THC-COOH	

Table VIII (continued). Comparison of DDA LC-MS-MS Procedure to Conventional GUS Technique (Immunoassay and REMEDi) of Urine Samples.

No.	Immunoassay	REMEDi	DDA LC-MS-MS	Comment/LC-MS Confirmation
7		Desmethylmirtazapine	Mirtazapine, Desmethylmirtazapine	No immunoassay
8		Venlafaxine, Desmethylvenlafaxine	Venlafaxine, Desmethylvenlafaxine	
	Benzodiazepine	Oxazepam	Oxazepam, Oxazepam-glucuronide,	
			Mirtazapine	
			Zolpidem	
			Metoprolol	
9	Antidepressant	Amitryptiline, Nortryptiline	Nortryptiline, E-10-OH-Nortryptiline	
	Benzodiazepine		Bromazepam, 3-OH-Bromazepam	
10	Opiate	Dihydrocodeine, Hydrocodone, Metabolite of Hydrocodone	Normorphine, Dihydrocodeine-glucuronide	Morphine, Dihydrocodeine, Heroin, 6-Acetylmorphine, Hydrocodone
			Zolpidem	
		Alpha-OH-Midazolam	4-OH-Midazolam	
			Phenprocoumon	
	Cocaine			Cocaine, Benzoylecgonine
11	Amphetamine	MDMA	Ephedrine, MDMA, Mephentermine	Ephedrine, MDMA, MDA, Amphetamine, Metamphetamine
	Cocaine	Benzoylecgonine	Benzoylecgonine	
	Benzodiazepine			
		Methadone, EDDP	EDDP	
			Lidocaine	
12	Methadone, EDDP	Methadone, EDDP	EDDP	
	Barbiturate (Phenobarbital)			
			Torasemide	
			Propylhexedrine	
13	Methadone, EDDP	Methadone, EDDP	Methadone, EDDP	
		Metabolite of chlorprothixene		Not in the library
	Cannabis			
14	Amphetamine	MDMA, MDA	MDMA	Immunoassay only Amphetamine
		Cocaine, Benzoylecgonine	Benzoylecgonine	
		Desethylflurazepam, Didesethylflurazepam	Flurazepam, N-OH-Ethylflurazepam, 3-OH-Bromazepam	Desethylflurazepam and Didesethylflurazepam not in the library
		Quinine		

Table VIII (continued). Comparison of DDA LC-MS-MS Procedure to Conventional GUS Technique (Immunoassay and REMEDi) of Urine Samples.

No.	Immunoassay	Remedi	DDA LC-MS-MS	Comment/LC-MS Confirmation
15	Cocaine		Benzoylecgonine	Cocaine, Benzoylecgonine
	Benzodiazepine	Temazepam, Desmethyldiazepam	Oxazepam, Temazepam, Temazepamglucuronide	
	Opiate			
	(Amphetamine)			Amphetamine negative
	Cannabis			
16		Olanzapine,	Olanzapine	
		7-OH-Quetiapine		7-OH-Quetiapine not in library
	Amphetamine	MDA, MDMA	MDA, MDMA, MDEA	MDA, MDMA, MDEA
	Benzodiazepine	N-OH-Ethyl-flurazepam		
		Carbamazepine	Carbamazepine	
17	n. d.	n. d.	n. d.	Ibuprofen and its metabolites (by HPLC-DAD)

Atracurium, dipyrone, fluconazole, bisoprolol, a metabolite of chlorprothixene and 7-OH-quetiapine were not detected because MS data of these substances were not recorded and included into the library. Venlafaxine, citalopram, tramadol, acetaminophen, diphenhydramine and quinine were not detected probably due to a higher LOD of the LC-MS-MS system. That means that the concentrations were in therapeutic concentrations and not in the toxic range. Heroin, 6-acetylmorphine and hydrocodone were also missed due to higher LOD of the DDA approach. Salicylate and phenobarbital were detected neither with the REMEDi system nor with the LC-MS-MS procedure. Other acidic substances like mefenamic acid, torasemide were identified only with the DDA method. Acidic substances can be detected with the presented HPLC-DAD method (see 4.1.). A typical HPLC-DAD chromatogram of mefenamic acid with its metabolites is shown (Fig. 13, case 37). Case 17 was a suspected intoxication with ibuprofen. Ibuprofen and its metabolites were only detected by the HPLC system.

The cut-off for the immunoassay test of cannabinoids is lower (50 ng/mL) than the LOD detected with the DDA approach. In cases (1, 5, 15) the concentrations of the 11-nor-delta-THC-COOH were above the cut-off of the immunoassay and below the LOD of the DDA approach, the substance was only identified by immunoassay. In higher concentrations (above the LOD) 11-nor-delta-THC-COOH was also detected by the LC-MS-MS system (in case 6).

In general the same substances were detected with both systems. With the developed LC-MS-MS system basic, neutral as well as acidic substances can be identified in the same run. The REMEDi is designed to identify basic and neutral drugs and their metabolites. Acidic substances like mefenamic acid cannot be detected with this system, benzodiazepines are analysed in a special second run (with other equipment). This is one of the major advantages of the DDA approach. Other advantages are that the analysis time is much shorter and the hydrolysis of glucuronides is not necessary. In addition samples often have to be diluted with the REMEDi system up to 3 - 5 times depending on the concentration of the substance.

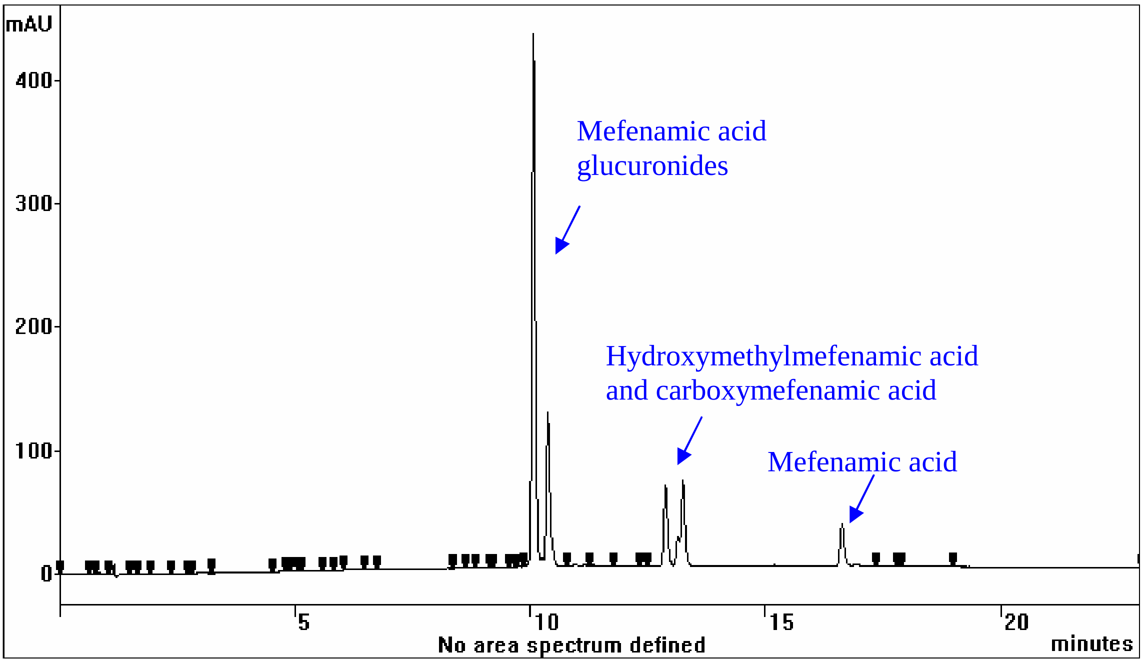


Fig. 13. Chromatogram of mefenamic acid and its metbolites of a serum sample (case 37).

The presented method is robust and the information content is high. Substances from different groups (amphetamines, antidepressants, benzodiazepines, cannabinoids, cocaine, opiates, antidepressant, diuretics, antidiabetics, opiates, neuroleptics, analgesics etc.) were detected and identified.

Table IX. Comparison of DDA LC-MS-MS Procedure to Conventional GUS Technique (Immunoassay and REMEDi) of Serum Samples.

No.	Immunoassay	REMEDi	DDA LC-MS-MS	Comment/LC-MS Confirmation
18	Benzodiazepine		Oxazepam, Oxazepam-glucuronide	No benzodiazepine REMEDi
		Moclobemide	Moclobemide	
19	Benzodiazepine		Oxazepam, Oxazepam-glucuronide	No benzodiazepine REMEDi
		Ranitidine	Ranitidine	
		Lidocaine, Metabolite of Lidocaine	Lidocaine	
		Atracurium		Not in the LC-MS-MS library
		Dipyrone		Not in the LC-MS-MS library
			Metoclopramide	
	Acetaminophen			
20	Methadone	Methadone	Methadone	
	Benzodiazepine		Temazepam, Oxazepam, Diazepam, Desmethyldiazepam	No benzodiazepine REMEDi
			Carbamazepine, 10,11-dihydro-10-OH-Carbamazepine, Oxcarbamazepine	
21	Opiate Alcohol	n. d.	n. d.	
22	Benzodiazepine		Oxazepam, Oxazepam-glucuronide, Desmethyldiazepam	No benzodiazepine REMEDi
		Metoclopramide	Metoclopramide	
23	n. d.	n. d.	n. d.	
24	Antidepressants	Desmethyltrimipramine	Trimipramine, Desmethyltrimipramine	
	Methadone	Methadone	Methadone	
	Benzodiazepine		Diazepam, Desmethyldiazepam	No benzodiazepine REMEDi
25	n. d.	Citalopram		Not found because of interference with clozapine
		Clozapine, Metabolite of Clozapine	Clozapine, N-Desmethylclozapine, Clozapine-N-Oxide	
			Amisulpiride	
			Atenolol	
26	Benzodiazepine	n. d.	Oxazepam, Oxazepam-lucuronide	No benzodiazepine REMEDi
			Gliclazide	

Table IX (continued). Comparison of DDA LC-MS-MS Procedure to Conventional GUS Technique (Immunoassay and REMEDi) of Serum Samples.

No.	Immunoassay	REMEDi	DDA LC-MS-MS	Comment/LC-MS Confirmation
27	Benzodiazepine		Lorazepam, N-OH-Ethylflurazepam, Desalkylflurazepam, Diazepam	No benzodiazepine REMEDi
		Sulpiride	Sulpiride	
		Venlafaxine		
		Metabolite of Venlafaxine		
		Tramadol		
			Lamotrigine	
28	n. d.	n. d.	n. d.	
29	n. d.	n. d.	n. d.	
30	Alcohol			
	Cananbis			
		Fluoxetine, Metabolite of Fluoxetine	Fluoxetine	
31	n. d.	Citalopram		
		Ephedrine?		
			Mefenamic acid	
32	Cocaine	Cocaine, Benzoylecgonine	Benzoylecgonine	
33	Benzodiazepine		Lorazepam	
34	Opiate	Pethidine, Metabolite of Pethidine	Pethidine	Metabolite not in the LC-MS-MS library
	Benzodiazepine		Diazepam	No benzodiazepine REMEDi
		Dipyrone		
35	Antidepressant	Amitryptiline	Amitryptiline, E-10-Amitryptiline, Nortryptiline	
		Zolpidem	Zolpidem	
	Salicylate			Ibuprofen and its metabolites (by HPLC-DAD)
36	n. d.	n. d.	n. d.	
37	n. d.	n. d.	Mefenamic acid	Mefenamic acid and its metabolites (by HPLC-DAD)
38	Opiate		Codeine-6-glucuronide	
	Cocaine	Cocaine, Benzoylecgonine	Benzoylecgonine	
	Acetaminophen			
39	n. d.	n. d.	n. d.	
40	n.d.	Fluconazole	n.d.	Not in the LC-MS-MS library
		Bisoprolol		Not in the LC-MS-MS library
41	n. d.	Quetiapine, 7-OH-Quetiapine	Quetiapine	Metabolite not in the LC-MS-MS library
			Lamotrigine	
42	n. d.	n. d.	n. d.	

5.4. CONCLUSION

In conclusion, for routine screening the combination of SPE, LC and APCI-MS represents an attractive alternative to the well-established technique of GC-MS. It was demonstrated in this study that the SPE-APCI-MS screening method is suitable for routine measurements of serum samples. The described procedure is fully automated (from the extraction to the detection of a drug) and easy to handle. The method is highly specific because compounds are detected and identified by their retention times, their molecular ions and fragments. Rapid identification in screening experiments was achieved by the creation of a small application program. With the method presented here, the analysis of a serum sample can be performed in less than one hour. The constructed library comprises more than 400 spectra with the corresponding relative retention times of more than 350 compounds.

Out of 100 tested compounds, only 13 were not detectable with this LC-MS method. These compounds have high serum concentrations and can easily be detected with HPLC-DAD. HPLC-DAD together with the described method seems to be an ideal combination for a GUS.

It was demonstrated that the DDA-mediated-LC-MS-MS screening approach was a valuable alternative to the traditional GUS procedure for the analysis of samples from potentially intoxicated individuals and drug addicts. Almost all of the drugs detected by the conventional techniques as well as additional drugs were identified.

The future approach will address the robustness of the method by analysing high numbers of samples from potentially poisoned patients and comparing to conventional screening methods. Furthermore, the quantification method of detected substances has to be established. It will also be of interest to expand the LC-MS-MS library.

6. APPENDIX

6.1. PROPOFOL

6.1.1. Introduction

Propofol (2,6-diisopropylphenol, I.C.I. 35868) is a rapid-acting, intravenously administered anaesthetic agent for the induction and maintenance of general anaesthesia (67).

For use in a clinical study (68), a fast and sensitive method was developed to determine propofol in human serum. Expected serum concentrations after propofol infusion were compared to real serum concentrations.

6.1.2. Materials and Methods

6.1.2.1. Reagents and Chemicals

Propofol and trimethylammonium chloride were obtained from Sigma-Aldrich Steinheim, Germany). All solvents and buffer solutions in HPLC grade were purchased from Merck (Darmstadt, Germany). Solid-phase extractions (SPE) were carried out on Oasis MCX cartridges (Waters, Rupperswil, Switzerland). Deionised water was prepared on a MilliQ Purification System.

6.1.2.2. Apparatus and Instrumental Conditions

A Varian Pro Star HPLC system was used for this analysis. It consisted of a Pro Star 230 solvent delivery module (SDM), a Pro Star 330 PDA Detector, and a Star chromatography workstation system with software version 5.50. The analytical column used was an Spheri-5, RP-8 (5 µm, 220 x 4.6 mm) protected by an Spheri-5, RP-8 (5 µm, 30 x 4.6 mm) both obtained from Perkin Elmer.

The HPLC was used in the isocratic mode with an aqueous mobile phase of 0.02 mol/L KH_2PO_4 buffer (pH 3.5) / acetonitrile (40:60, v/v) at a flow rate of 1.2 mL/min. The photodiode array (PDA) detector was set at a detection wavelength of 220 nm, and the injection volume was 150 µL in a loop of 20 µL. The approved method resulted in a retention time of 9.9 min for propofol and an overall running time of 15 min.

6.1.2.3. Standard Preparation

A stock solution of propofol (100 mg/mL) was prepared in acetonitrile/water (20:80, v/v). The working standards were made from the stock by appropriate dilutions with deionised water. The solutions were added to lyophilized drug free serum (Biorad, Reinach, Switzerland) yielding concentrations ranging from 0.1 to 10 mg/L.

6.1.2.4. Extraction Procedure

The solid-phase extraction (SPE) procedure was carried out on Oasis MCX cartridges. The Oasis MCX cartridges were conditioned initially with acetonitrile 1ml, followed by 1 ml 0.1 mol/L phosphate buffer (pH 9.1). 1 mL per serum sample was loaded onto the cartridges. Cartridges were subsequently washed with 1 mL 0.1 mol/L phosphate buffer (pH 9.1) and dripped dry under charging pressure. The compound of interest was eluted with 1 mL acetonitrile and aliquots were injected into the HPLC columns.

6.1.3. Results and Discussion

The absolute recoveries of propofol in serum were determined by quantitating the measured amount over the range of 0.1-20 mg/L compared to primary standards directly made up in acetonitrile/water (Table X). The absolute recovery was between 91 and 116%. This absolute recovery illustrates good extraction efficiency.

Table X. Absolute Recoveries of Propofol in Serum Compared to Propofol Standards in Acetonitrile/Water.

Range studied (mg/L)	Absolute recovery in serum (%)	
	Mean	CV
0.1	100	16.3
1	101	3.9
5	116	7.5
10	92	5.8
20	91	3.5

The limit of quantification of the procedure was found to be 0.1 mg/L based on the signal to noise response and the standard deviation of samples in serum. The procedure showed good linearity between the concentrations of 0.1 and 10 mg/L with a correlation coefficient of 0.997.

On eight different days 2 samples were quantified of each concentration. Intraassay coefficients of variation (CV) at the concentrations of 0.1, 1.0 and 10 mg/L were 11.6%, 2.8% and 3.0%, respectively. Interassay CV at the concentration of 0.1, 1.0 and 10 mg/L were 16.0%, 9.2% and 4.4% (Table XI), respectively.

Table XI. Validation Data for Propofol.

Concentration (mg/L)	Coefficients of variation (%)	
	Intraassay	Interassay
0.1	11.6	16.0
1	2.8	9.2
10	3.0	4.4

For a clinical study (GLP certified) propofol concentrations were measured in serum samples of 9 different individuals. Fig. 14 shows the serum concentration-time curves of propofol after intravenous administration of propofol. With an automated system propofol was individually administered to the subjects according to a fix dosis scheme. From 45 to 105 minutes the serum concentration was expected to be at 1 mg/L, from 120 to 180 minutes at 2 mg/L, and finally from 195 to 255 minutes at 3 mg/L. At each time point serum was taken and analysed.

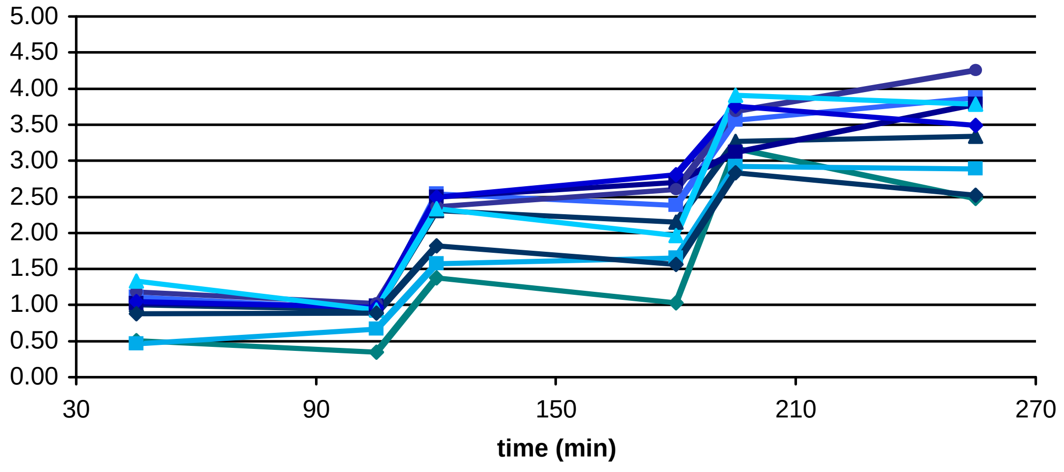


Fig. 14. Serum concentration-time curves of propofol after intravenous administration of propofol to 9 individuals (P01-P09). From 45 to 105 minutes the propofol serum concentration was expected to be at 1 mg/L, from 120 to 180 minutes at 2 mg/L and from 195 to 255 minutes at 3 mg/L.

Expected serum concentrations after propofol infusion were compared to real serum concentrations. The measured serum concentrations matched well with the expected serum concentrations (Table XII). The difference of the average of the 9 individuals between the measured and the expected propofol serum concentrations were less than 15% at each time point.

Table XII. Average of Propofol Serum Concentrations of Nine Individuals after Infusion. Difference between the Expected and the Measured Serum Concentrations.

Time (min)	Expected serum concentration mg/L	Measured serum concentration mg/L		Difference (%) between measured and expected concentration
		Mean	S.D.	
45	1	0.95	0.29	-5
105	1	0.85	0.21	-15
120	2	2.15	0.44	+8
180	2	2.09	0.60	+5
195	3	3.36	0.39	+12
255	3	3.38	0.62	+13

The method proved to be reproducible, accurate, simple and useful in clinical investigation and in monitoring propofol concentrations. The aimed serum concentrations of propofol in the clinical study were achieved with less than 15% difference at each time point.

6.2. REMIFENTANIL

6.2.1. Introduction

Remifentanil is an ultra-short-acting opioid anaesthetic agent. It is extensively used for short-term anaesthesia, often in combination with sedatives. The compound has a N-substituted labile methyl ester which is highly susceptible to chemical and enzymatic hydrolysis resulting in a short half-life of remifentanil (69). It has an elimination half-life in vivo of 3-10 min, which is much shorter than those of fentanyl, sufentanil and alfentanil. The addition of citric acid lowers the pH sufficiently to prevent forms of hydrolysis, allowing blood samples to be safely stored for at least 20 h at room temperature, frozen for at least a year (70).

To date, bioanalytical literature for the chromatography of remifentanil has described a GC-MS method (71), a GC method with nitrogen-specific detection (70), 2 HPLC methods with UV detection (69, 72) and a LC-MS-MS method (73). The HPLC are simpler and less expensive, but have limited sensitive. They were not appropriate for our pharmacokinetic study. The described GC-MS methods require a time consuming sample preparation and analytical procedure. As extraction procedure all the methods used a LLE (69, 72). The combination of sensitivity, specificity and high sample throughput were the main reasons to use LC-MS-MS with SPE.

For use in a clinical study (68), a fast and sensitive assay method was developed using a SPE to determine remifentanil in human whole blood samples.

6.2.2. Materials and Methods

6.2.2.1. Reagents and Chemicals

Remifentanil (GI87084) and its main metabolite (GI90291A), an acid derivative, were obtained from Glaxo Smith Kline (Geneva, Switzerland). 6-Acetylcodeine was obtained from Lipomed (Arlesheim, Switzerland). All the other reagents and solvents in HPLC grade were purchased from Merck (Darmstadt, Germany). Solid-phase extractions were carried out on Oasis HLB cartridges (Waters, Rupperswil, Switzerland). Deionised water was prepared on a MilliQ laboratory purification system.

6.2.2.2. Apparatus and Instrumental Conditions

The LC-MS-MS apparatus was a LCQ Advantage from Thermo Finnigan (Allschwil, Switzerland) equipped with an ESI device operating in the positive detection mode. Chromatography was performed on a Symmetry C_8 column (2.1 x 150 mm, 5 µm) from Waters (Rupperswil, Switzerland). The mobile phase was delivered at a flow rate of 200 µL/min. Each chromatographic run was performed with a binary, linear A/B gradient (solvent A 0.1% formic acid, solvent B acetonitrile). Elution began with the mobile phase at 12% B and 88% A for 1 min, then a linear gradient to percentage of 80% B over 6 minutes. The percentage of B was decreased back to 12% for 3 minutes. For quantification, the selected ion monitoring mode was used to obtain the desired sensitivity. Table XIII lists the precursor and product ions and relative collision energy for each analyte. The heated capillary temperature was 350°C, capillary voltage 3.8 kV, sheath gas (nitrogen) flow was 60 units.

Table XIII. Precursor and Main Product Ions and Relative Collision Energies of Remifentanil, its Metabolite and IS.

Compounds	Precursor ion (m/z)	Full scan MS-MS (amu)	Main product ions used for quantification (m/z)	Collision energy (%)
remifentanil	377.2	250-385	285.3, 317.1, 344.9	35
Main metabolite	363.1	200-400	259.3, 302.8, 330.8	35
6-acetyl-codeine	342.1	200-400	225.0, 282.0	35

6.2.2.3. Standard Preparation

Stock solutions (1 mg/mL) of remifentanil and its metabolite were prepared in acetonitrile containing 0.1% formic acid. The stock solutions were diluted further with 0.1% formic acid. Calibration standards were prepared at different concentrations in the range of 0.5 to 10 ng/mL. Appropriate amounts of diluted solutions of remifentanil and its metabolite were added to tubes containing 1 mL heparinised whole blood and 20 µL of 50% w/w citric acid. 6-Acetylcodeine was prepared as internal standard (IS) at a concentration of 100 ng/mL.

6.2.2.4. Extraction Procedure

After blood sample collection it is imperative that enzymatic degradation of remifentanil is stopped immediately. 20 µL of 50% w/w citric acid were added to 1 mL heparinised whole blood to prevent hydrolysis of remifentanil via pH control and were freezed until the day of analysis. 100 μL of internal standard solution were pipetted to each sample. To precipitate proteins the samples were first vortexed with 1 mL of methanol, then with 1 mL 0.174 M $ZnSO_4$-solution and finally with 2 mL 0.035M $ZnSO_4$-solution. Then the samples were shaken for 20 minutes. The mixture was then centrifuged for 10 min at 4'500 rpm. The supernatants were diluted with water to 10 mL.

The Oasis HLB extraction cartridges were conditioned with 1 mL methanol, and with 1 mL water. 9.9 mL of the solution was loaded on the cartridge. The sorbent was washed with 1 mL water, and eluted with 1 mL methanol. A new cartridge was used for each sample. Eluents were evaporated to dryness under a stream of dry nitrogen. The extracts were reconstituted in 100 μL acetonitrile/0.1% formic acid (10:90, v/v) and aliquots were injected into the HPLC columns.

6.2.3. Results and Discussion

The retention times of the compounds of interest were 5.2 min for 6-Acetylcodeine, 5.4 min for remifentanil and 5.5 min for the metabolite of remifentanil (Fig. 15). Table XIV shows the coefficients of variation (CV) for the intraday and the interday precision of remifentanil and its metabolite.

On eight different days 2 samples were quantified of each concentration. The CVs for the intraday precision were always less than 15% for remifentanil and its metabolite. CVs for remifentanil for the interday precision were 18.0%, 10.2% and 9.7% at 0.5, 2 and 10 ng/mL, respectively. For the metabolite of remifentanil they were equal to 15.0%, 11.9%, 8.9% for the same concentrations and respectively.

Table XIV. Validation Data for Remifentanil and its Metabolite.

Concentration (ng/mL)	**CV % of Remifentanil**		**CV % of the Metabolite of Remifentanil**	
	Intraday	**Interday**	**Intraday**	**Interday**
0.5	12.6	18.0	14.3	15.0
2	9.1	10.2	9.1	11.9
10	8.8	9.7	11.2	8.9

The absolute recoveries of remifentanil and its metabolite in whole blood were determined by quantitating the amount recovered against standards made up directly in acetonitrile/water.

The overall remifentanil recovery was 78% and 76% for the metabolite of remifentanil. The procedure showed good linearity between the concentrations of 0.5 to 10 ng/mL.

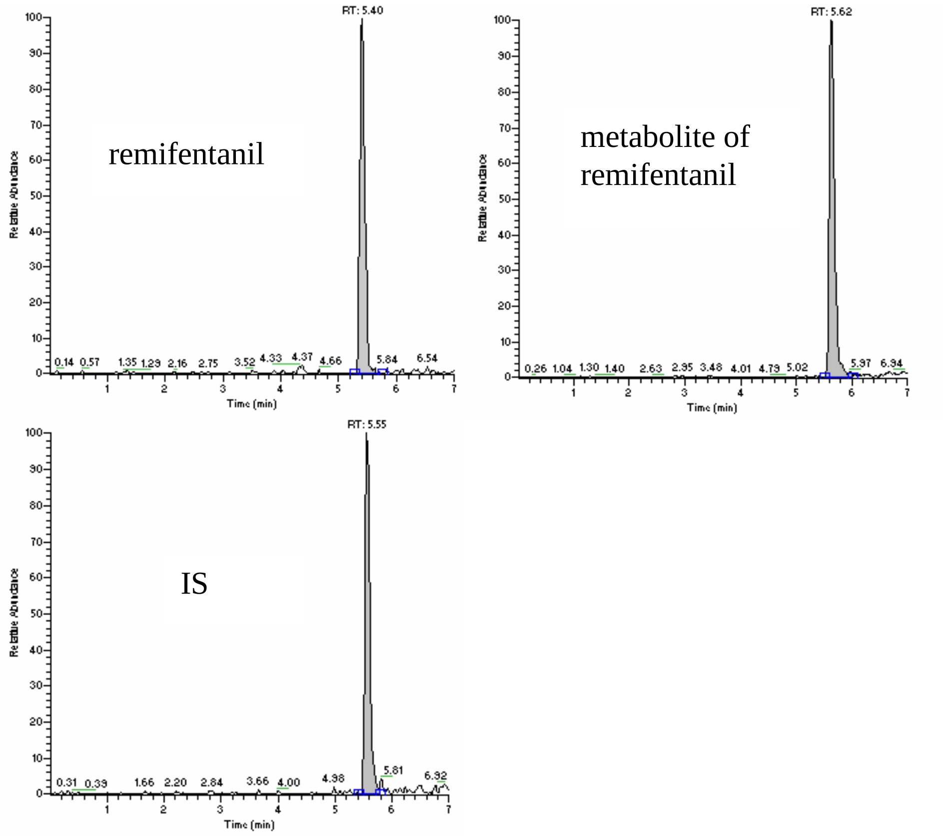


Fig. 15. Representive chromatogram of an extracted whole blood sample of a test person at 1.96 ng/mL remifentanil, 6.38 ng/mL metabolite of remifentanil and IS.

Fig. 16 and 17 show the whole blood concentration-time curves of remifentanil and its metabolite, respectively, after intravenous administration. With an automated system remifentanil was individually administered to the subjects according to a fix scheme.

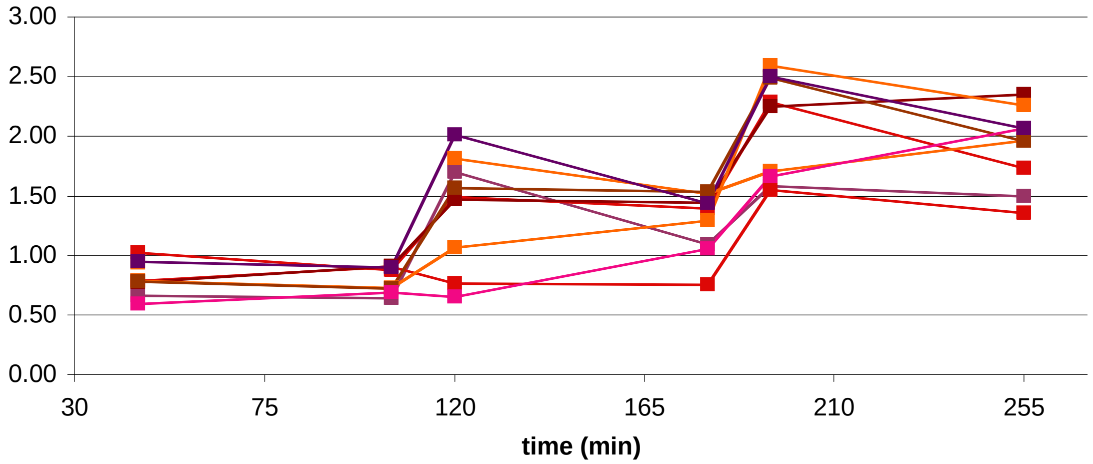


Fig. 16. Whole blood concentration-time curves of remifentanil after intravenous administration of remifentanil to 9 individuals (P01-P09). From 45 to 105 minutes the remifentanil whole blood concentration was expected to be at 1 ng/mL, from 120 to 180 minutes at 2 ng/mL and from 195 to 255 minutes at 3 ng/mL. Blood samples were taken after 45, 105, 120, 180. 195 and 255 minutes.

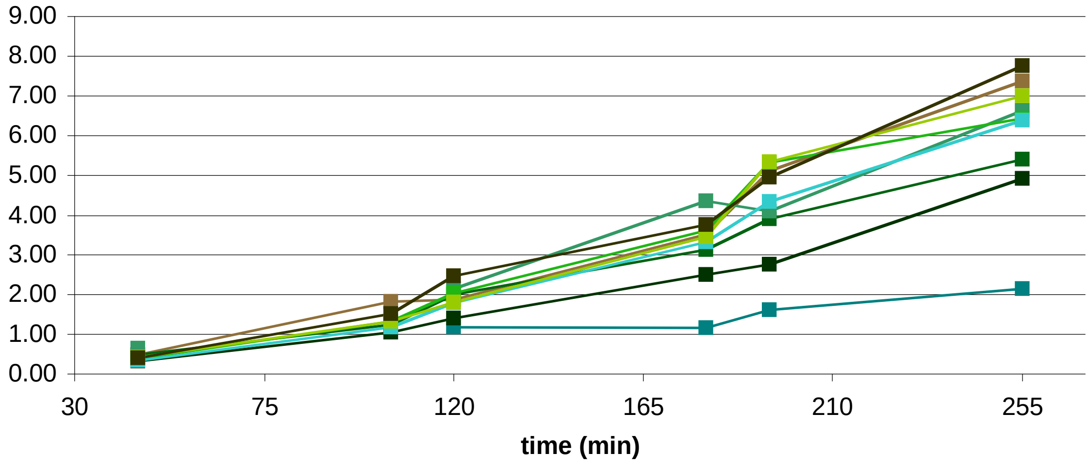


Fig. 17. Whole blood concentration-time curves of the main metabolite of remifentanil after intravenous administration of remifentanil to 9 individuals.

From 45 to 105 minutes the whole blood concentration was expected to be at 1 ng/mL, from 120 to 180 minutes at 2 ng/mL, finally from 195 to 255 minutes at 3 ng/mL. At different time points whole blood samples were taken and analyzed.

Expected whole blood concentrations after remifentanil infusion were compared to real whole blood concentrations. The measured whole blood concentrations were all much deeper than the aimed whole blood concentrations (Table XV). The difference between the expected and the measured whole blood concentration were up to –54%.

Table XV. Average of Remifentanil Whole Blood Concentrations of Nine Individuals after Infusion. Difference between the Expected and the Measured Whole Blood Concentration.

Time (min)	Expected whole blood concentration ng/mL	Measured whole blood concentration ng/mL		Difference (%) between measured and expected concentration
		Mean	**S.D.**	
45	1	0.81	0.14	-19%
105	1	0.79	0.11	-21%
120	2	1.39	0.44	-31%
180	2	1.28	0.27	-36%
195	3	2.07	0.43	-47%
255	3	1.92	0.35	-54%

A sensitive and specific assay for the determination of remifentanil and its metabolite in human whole blood using LC-MS-MS has been developed. The method has a validated range for remifentanil and its metabolite from 0.5 to 10 ng/mL and has been used to analyse clinical blood samples in a GLP certified study.

6.3. APOMORPHINE

6.3.1. Introduction

Apomorphine is indicated for the treatment of erectile dysfunction. LC-MS methods have not yet been published for the assessment of apomorphine in plasma. For use in a clinical study, a fast and sensitive assay method was developed to determine apomorphine in human plasma samples.

6.3.2. Materials and Methods

6.3.2.1. Reagents and Chemicals

Apomorphine and 6-acetylcodeine was obtained from Lipomed (Arlesheim, Switzerland). All the other reagents and solvents in HPLC grade were purchased from Merck (Darmstadt, Germany). C18 EC cartridges were purchased from Spark Holland (Emmen, Netherlands). Deionised water was prepared on a MilliQ laboratory purification system.

6.3.2.2. Apparatus and Instrumental Conditions

The LC-MS-MS apparatus was a LCQ Advantage MAX from Thermo Finnigan (Allschwil, Switzerland) equipped with an ESI device operating in the positive detection mode. Chromatography was performed on a Phenomenex Synergi MAX-RP column (2.0 x 75 mm, 4 µm) from Brechbuehler (Schlieren, Switzerland). The mobile phase was delivered at a flow rate of 500 µL/min.

Each chromatographic run was performed with a binary, linear A/B gradient (solvent A 0.1% formic acid, solvent B acetonitrile with 0.1% acetonitrile). Elution began with the mobile phase at 5% B and 95% A for 1.5 min, then a linear gradient to percentage of 90% B over 3.5 minutes. The percentage of B was held for 2.2 min decreased back to 5% for 3.8 minutes.

For quantification, the selected ion monitoring mode was used to obtain the desired sensitivity. Table XVI lists the precursor and product ions and the normalized relative collision energy for each analyte. The heated capillary temperature was 350°C, ionization voltage 4.5 kV, sheath gas (nitrogen) and auxiliary gas flow were 80 and 15 relative units, respectively.

Table XVI. Precursor and Main Product Ions and Relative Collision Energies of Apomorphine and IS.

Compounds	Precursor ion (m/z)	Main product ions used for quantification (m/z)	Normalized collision energy (%)
apomorphine	268.3	218.7, 236.7, 237.7	35
6-acetylcodeine	342.1	225.0, 282.0, 342.0, 343.0	35

6.3.2.3. Sample Preparation

Stock solutions (0.1 mg/mL) of apomorphine were prepared in methanol. The stock solutions were diluted further deionised water. Calibration standards were prepared at different concentrations in the range of 1 to 30 ng/mL. Appropriate amounts of diluted solutions of apomorphine were added to plasma. 6-Acetylcodeine was prepared as internal standard (IS) at a concentration of 2000 ng/mL.

6.3.2.4. Extraction Procedure

100 μL of internal standard solution were pipetted to 1 ml of plasma of each sample. On-line SPE and elution was performed using the Prospekt 2 system. The C18 EC cartridge was conditioned with 1 mL methanol (5 mL/min) and with 1 mL water (5 mL/min). 100 μL of the plasma mixture was loaded on the cartridge. The sorbent was washed with 1 mL water (2 mL/min), and eluted with the mobile phase during 10 minutes.

6.3.3. Results and Discussion

Representive chromatograms of a standard sample are shown at 1 ng/ml. The retention times of the compounds of interest were 3.7 min for apomorphine, and 3.9 min for the IS (6-acetylcodeine) (Fig. 18).

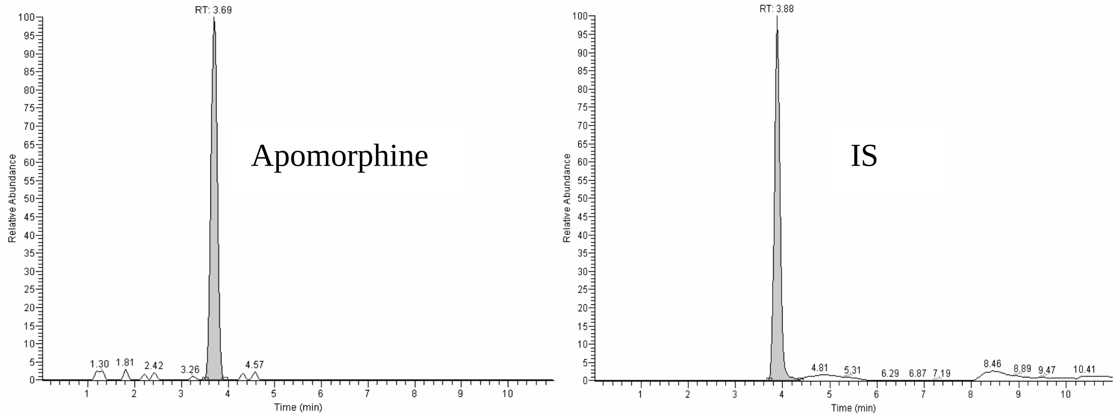


Fig. 18. Representive chromatogram of a standard serum sample at 1 ng/mL apomorphine (left) and IS (right).

Table XVII shows the coefficients of variation (CV) for the intraday and the interday precision of apomorphine. On six different days 2 samples were quantified of each concentration.

Table XVII. Validation Data for Apomorphine.

Concentration (ng/mL)	CV % of apomorphine	
	Intraday	Interday
2	11.6	11.4
10	6.8	8.8
20	4.5	7.4

The CVs for the intraday and interday precision were always less than 15% for apomorphine. CVs for apomorphine for the interday precision were 11.6%, 6.8% and 4.5% for the interday precision 11.4%, 8.8%, 7.4% at 2, 10 and 20 ng/mL, respectively.

The procedure showed good linearity between the concentrations of 0.5 to 30 ng/mL with a correlation coefficient of 0.991 (Fig. 19).

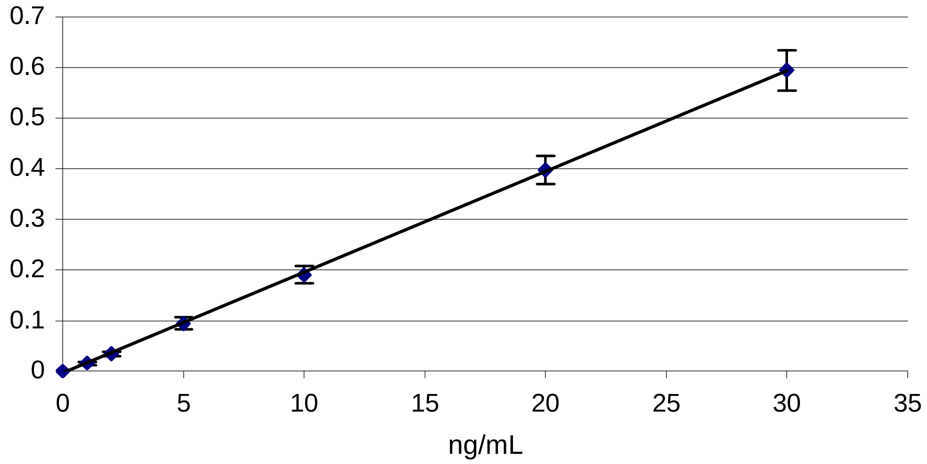


Fig. 19. Validation data for apomorphine.

Two different formulations of tablets with apomorphine were ingested. At different time points plasma samples were taken and analyzed (30 min, 15 min, 5 min before, and 15 min, 30 min, 45 min, 60 min, 90 min, 120 min, 180 min, 240 min and 360 min after ingestion of a tablet). Fig. 20 shows the plasma concentration-time curves of apomorphine.

In determining bioequivalence, pharmacokinetic studies were conducted on each of the preparations in volunteer subjects. The bioequivalence of a formulation of a generic apomorphine compared to the reference product (Uprima®) was assessed in a study.

Preparations are bioequivalent if the 90% confidence intervals (90% CI) between the two preparations, of c_{max} and AUC lie in the range 0.80-1.25. t_{max} was considered because the onset time is therapeutically relevant.

Plasma samples were obtained at regular intervals and assayed for drug concentration. This data was used to assess key pharmacokinetic parameters such as area under the curve (AUC), peak concentration (c_{max}) and time to peak concentration (t_{max}).

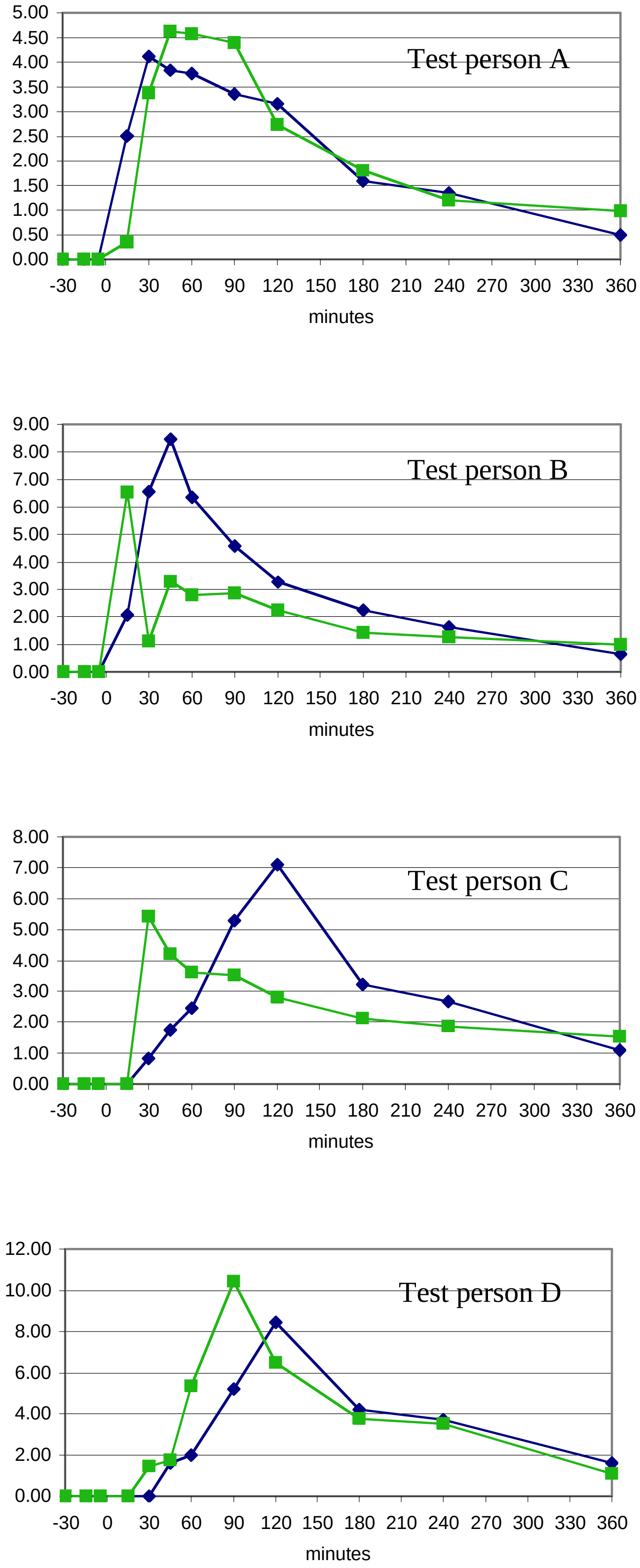


Fig. 20. The plasma concentration-time curves of apomorphine are shown in two different formulations after tablet administration. The concentration of the Uprima® formulation is displayed in blue colour, the generic formulation in green. At different time points plasma samples were taken and analyzed.

Table XVIII. Half-life and c_{max} of Apomorphine of 4 Different Test Persons in the Original Product and a Generic Formulations.

Proband	**$t^{1}/_{2}$ of apomorphine (min)**		**c_{max} of apomorphine (ng/mL)**	
	Uprima®	**Generic formulation**	**Uprima®**	**Generic formulation**
P01	100	124	4.1	4.6
P02	95	107	8.5	6.5
P03	94	144	7.1	5.4
P04	107	89	8.4	10.4
mean	99	116	7.0	6.7
CV	6	24	2.1	2.6

Table XVIII and XIX show the half-life, c_{max}, t_{max} and AUC of apomorphine in 4 different probands in the original product (Uprima®) and in the generic formulation. Most of the parameters were similar between the 4 test persons. The mean of the AUC of the generic formulation was within the range of 0.80-1.25 compared to the mean of the AUC of the original product. The other pharmokinetic parameters ($t^{1}/_{2}$, c_{max}, t_{max}) of apomorphine didn't differ significantly by the administration of the generic formulation. $t^{1}/_{2}$ was 99 and 116 minutes, c_{max} was 7.0 and 6.7 ng/mL, t_{max} was 79 and 45 minutes for the original and the generic formulation, respectively.

Table XIX. t_{max} and AUC of Apomorphine of 4 Different Test Persons in the Original Product and a Generic Formulations.

proband	**t_{max} of apomorphine (min)**		**AUC ($h*mg/L*10^{-3}$)**	
	Uprima®	**Generic formulation**	**Uprima®**	**Generic formulation**
P01	30	45	13.4	15.5
P02	45	15	18.2	13.8
P03	120	30	20.3	19.4
P04	120	90	25.6	25.3
mean	79	45	19.4	18.5
CV	48	32	5.1	5.1

Further studies have to be performed. 4 different test persons are not sufficient to prove bioequivalence. Usually, 18 to 24 subjects are required to perform a bioequivalence study for generic drug development.

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