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Methodology for the development of a drug library based upon collision-induced fragmentation for the identification of toxicologically relevant drugs in plasma samples

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Abstract

The possibility of creating a robust mass spectral library with use of high-performance liquid chromatography–atmospheric pressure–electrospray ionization (HPLC–AP–ESI) for the identification of drugs misused in cases of clinical toxicology has been examined. Factors reported as influencing the fragmentation induced by ''source transport region collision induced dissociation'' (CID) have been tested in this study (i.e. solvent, pH, different acids or buffer salts and their concentration, different organic modifiers and the modifier concentration). The tests performed on a few ''model drugs'' were analysed with use of two different single quadrupole instruments. The large number of mass spectra obtained appears to be affected by the mobile phase conditions to only a minor extent. This also holds for the mass spectra obtained at two different instruments (laboratories). Subsequently breakdown curves have been measured for about 20 randomly chosen drugs by variation of the kinetic energy of their ions in the CID zone through changing the fragmenter voltage. These breakdown curves were used to optimize the fragmenter voltage for each drug. The optimized fragmenter voltages were then applied by use of a variably ramped fragmenter voltage to acquire mass spectra for the library. The chromatographic separations were run on a Zorbax Stable bond column using a 10-m*M* ammonium formate–acetonitrile gradient method. Spiked blank serum and patient samples with a total of 40 different drugs were extracted with use of a standard basic liquid–liquid extraction (LLE) method. A search of significant peaks in the chromatogram by application of the developed mass spectral library is shown to result in a more than 95% positive identification. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Collision-induced dissociation; Drugs screening; Library searching

1. Introduction

In clinical and forensic cases an efficient and extensive drug screening procedure is essential to either exclude the involvement of drugs and poisons, or detect such substances should they be present. The presence of acidic, basic or neutral properties in drugs and the overall drug lipophilicity affect the ability to extract such substances from biological

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matrices, while thermal stability, polarity and detector sensitivity affect the detectability of drugs in chromatographic systems [1].

The ability to perform a comprehensive and systematic analysis of specimens with regard to the presence of chemicals in concentration of toxicological importance, is termed systematic toxicological analysis (STA). Above this concentration the drug may give an expression of toxicity.

Most of the STA methods available in the literature are based on either HPLC–UV or GC–MS [2–11]. Both techniques have some drawbacks. HPLC–UV spectra are in general influenced by the solvent, pH and ionic strength, in which the compounds are dissolved [5,9].

This makes the identification of unknown drugs unreliable. GC–MS has weak points as well as a separation/identification technique compared to HPLC–MS. First, it is unsuitable for the direct analysis of polar compounds. Second, this technique is unsuitable for non-volative, thermolabile and high mass molecules [12].

The coupling of high-performance liquid chromatography with atmospheric pressure ionization mass spectrometry (HPLC–AP–MS) in electrospray is a breakthrough for its application to biomedical chromatography and toxicology. Since the last few years a very rapid development and a growing application of these techniques for the determination of toxicologically relevant compounds are observed [13–20]. HPLC–AP–MS has become a real alternative for GC–MS, particularly for the dedicated assay of polar drugs and their metabolites and large thermolabile molecules, which are inaccessible for gas chromatography without derivatization, like morphine glucuronides, LSD or polar cocaine metabolites [21–31]. However, GC–MS shows one very important advantage; the electron ionization (EI)-generated mass spectra are very reproducible and several databases have been developed for identification purposes. These databases are generally commercially available together with GC–MS instruments.

The use of HPLC–AP–MS becomes more widespread and consequently the need for searchable libraries becomes also more apparent. However, the inter- and intralaboratory reproducibility of HPLC–AP–MS mass spectra, which are a prerequisite for setting up a spectrum library, are until now not well known or checked without success. From anecdotal information one might suspect that API–LC–MS generated spectra are laboratory- or instrument-specific. Also, some authors have reported that ESI mass spectra may change when recorded in time, even when the batch of mobile phase of the same composition is unchanged [13,32].

Apart from EI, fragmentation of organic compounds is readily achieved by energetic collisions in the ESI source and can be induced by increasing the skimmer nozzle, cone, capillary, and orifice voltage, respectively, depending on the construction of the source.

In the instrument used, CID was induced by varying the voltage between the capillary exit and the skimmer nozzle. For this reason, the name CID in the transport region is used [33].

In the present study the following goals have been pursued:

- Examine the influence of different mobile phase conditions on the mass spectra.
- Check the similarity of spectra collected with two different instruments (same brand, but different type) in different laboratories.
- Identify the optimal fragmentation voltage on the basis of breakdown curves.
- Optimize a generic HPLC–AP–ESI–MS method for the analysis of STA.
- Collect an AP–ESI–MS library for the use of identification of unknown drugs of abuse and check if during library search the ''unknown'' compounds are identified.

2. Experimental

2.1. Reagents

All tested drugs were obtained from the hospital pharmacy of the ''Onze Lieve Vrouwe Gasthuis'' in Amsterdam, The Netherlands. The solvents used were of HPLC quality obtained from J.T. Baker (Deventer, The Netherlands). Acetic acid and formic acid, both p.a. grade, were obtained from Merck (Darmstadt, Germany). Ammonium formate, analytical grade, was supplied by Sigma–Aldrich (Steinheim, Germany) and ammonium acetate, also

analytical grade, was obtained from Merck (Darmstadt, Germany).

The blank plasma used for the method validation was obtained from a local blood bank and was preliminarily screened for the absence of drugs using an enzyme multiplied immunoassay technique (EMIT).

Plasma samples were obtained as positive toxicological samples in the hospital pharmacy of the ''Onze Lieve Vrouwe Gasthuis'' in Amsterdam.

2.2. Preparation of the standard solutions

Stock standard solutions, with a concentration 100 times the concentration at the toxicological level (Table 4), were prepared in methanol.

The standard solutions were stored in the dark at room temperature. Working solutions were prepared by diluting the stock standard solution 100 times with methanol. Spiked samples were prepared by adding 10 μl of the stock solution to 1 ml human blank plasma.

2.3. Sample preparation

During the testing period two different sample preparation techniques were used. These are standard liquid–liquid extraction methods (1). A buffer solution was prepared by dissolving 4.2 g $NaHCO_3$ and 2.3 g NaOH, both p.a. grade and supplied by Merck (Darmstadt, Germany), in 500 ml de-ionized water. From this buffer solution with a pH of 11, 1 ml was added to 1 ml of the serum–plasma sample in a round bottom glass centrifuge tube which can be closed with a stopper, and mixed on a vortex mixer. To this solution, 6 ml of a mixture of diethyl ether–dichloromethane (2:1, v/v) were added and the centrifuge tube was closed with a stopper.

This mixture was mixed for 5 min on a vortex mixer. The centrifuge tubes were centrifuged for 10 min at 2500 rpm. The supernatant was transferred to a clean glass tube and dried at maximum 40°C under nitrogen. The residue was dissolved in 100 μl methanol and injected into the HPLC. For the acid extraction, only the buffer was replaced by an acid buffer consisting of 10 g KH_2PO_4 (p.a. grade, Merck, Darmstadt, Germany), which was adjusted with phosphoric acid at pH 2.5. The rest of the procedure was like before.

2.4. Liquid chromatography

A liquid chromatograph (Agilent™, model 1100, Waldbronn, Germany) equipped with a binary pump (G1312A) with degassing unit (G1322), autosampler (G1313A), thermostatted column compartment (G1316A) and a diode array detector (G1315A) was connected to a mass selective detector. The set of experiments done for testing the influence of the mobile phase content on the mass spectrum was performed by automatic flow injection analysis (FIA). Tests were made with different concentrations of methanol or acetonitrile as organic phase and different concentrations of acetic acid, formic acid, ammonium acetate and ammonium formate as buffer solution. Flows were varied between 0.5 and 1.0 ml/min. The injection volume used during the FIA analysis was 10 μl. The chromatographic separation was performed during a gradient run on a 25-cm Zorbax SB C_{18} column with an I.D. 4.6 mm and 5 μm particles (P/N 880975.902). The mobile phase consisted of 10 m*M* ammonium formate adjusted with formic acid to pH 2.9 and acetonitrile containing 0.05% formic acid, the latter preventing pH changes during the elution which results in stable retention times. The flow was 0.7 ml/min. The gradient started with 90% 10 m*M* ammonium formate at pH 2.9 and 10% acetonitrile (0.05% formic acid). Linearly from time 0 min the concentration of acetonitrile is increased to 60%. From 10 to 24 min the concentration of acetonitrile is increased linearly to 80% and from 24 to 26 min linearly to 100%, where it stays until the stop time of 30 min. The equilibration time between injections was 7 min. The column was thermostatted at a temperature of 40°C. Injection volume for standard and samples was 25 μl.

2.5. AP–ESI–MS

A G1946A single quadrupole mass selective detector (Agilent Technologies, Palo Alto, CA, USA) equipped with an AP–ES source was used in the ''Onze Lieve Vrouwe Gasthuis'' in Amsterdam, The Netherlands. Some comparing tests were performed

in the application laboratory from Agilent in Amstelveen, The Netherlands. The instrument used there, was a G1946B single quadrupole mass selective detector. The G1946B has a different electronic functionality. The G1946B is advanced with the possibility to acquire and analyse four signals in parallel (for example, acquire a positive and a negative signal during one run). Both systems were controlled by a chemstation (version A 07.01) including the NIST mass spectra search program (version 1.6b). Both systems were equipped with an autotune algorithm used for the calibration of the mass analyzer. Before acquisition of a series of analyses a ''check-tune'' was performed to check the calibration of the mass analyzer. All tests, unless specifically mentioned, were performed in the ESI positive mode. The ESI parameters were optimized, using FIA of paracetamol and amitriptyline. The value, leading to the highest ion abundance, was used for the rest of the experiments. This resulted in the following settings of the AP–ESI conditions: nebulizer gas 55 p.s.i., drying gas 11 l/min., drying gas temperature 300°C and V_{cap} 3500 V.

Mass spectra of substances were taken in scan mode between 50 and 500 a.m.u. (step size 0.25, chromatographic PWHH, i.e. a peak width at half height of 0.20 min) at a detector gain of 6 and a threshold of 20. In the negative mode, only the polarity was changed.

2.6. Influence of the organic modifier concentration on the mass spectra

Experiments to test the influence of the organic modifier properties were performed with different concentrations of acetonitrile and methanol, for each being 10, 25, 50, 75 and 90%. Tests were performed with five different inorganic mobile phases; 0.1% acetic acid, 0.1% formic acid, 10 m*M* ammonium formate (pH 3), 10 m*M* ammonium acetate (pH 6).

First paracetamol and amitriptyline were used as test drugs, but later on also codeine, midazolam and amphetamine. From these drugs 10 μl of the working solution were injected individually by FIA.

The mass spectra were obtained with a varying fragmenter voltage (60, 80, 100, 120, 160 and 240 V) for each drug. This was repeated for every solvent composition.

2.7. Influence of the buffer molarity and pH on the mass spectra

The experiments were performed using formic acid in three concentrations (0.1, 0,01 and 0.001%), acetic acid in two concentrations (0.1 and 0.05%), ammonium acetate buffer at two concentrations (10 and 100 m*M* at pH 6) and ammonium formate buffer (10 and 100 m*M* at pH 3).

As organic modifiers acetonitrile and methanol were used. The same set-up was applied as in the testing of the influence of the organic modifier concentration. The experiments were carried out with FIA injections. The mass spectra were obtained at different fragmenter voltages (60, 80, 100, 120, 160 and 240 V) for each drug.

2.8. Influence of flow speed on mass spectra

The experiments were performed using 0.1% acetic acid and five different concentrations of the organic modifier methanol (10, 25, 50, 75 and 90%). The flows tested were 0.5 and 1.0 ml/min. The same general set-up was used as in the experiments described above. Paracetamol and amitriptyline were used as test compounds. The mass spectra were obtained at different fragmenter voltages (60, 80, 100, 120, 160 and 240 V).

2.9. Influence of inter-instrumental factors on mass spectra

Two instruments, which were described above, have been used for the experiments. The tests to compare the mass spectra, acquired on the two different instruments, were carried out with 10 m*M* ammonium formate, 0.05% acetic acid and 0.05% trifluoracetic acid as inorganic buffer.

The organic modifier methanol or acetonitrile was used in different concentrations (90, 75, 50, 25 and 10%).

The instrumental set-up was the same as mentioned before and paracetamol, amitriptyline and codeine were used as test compounds.

Both mass spectrometers were calibrated by performing an autotune prior to acquisition of the mass spectra.

2.10. Optimization of the fragmenter voltage for library generation

For every tested drug, the fragmenter voltage was determined at which the ''best'' mass spectral data, with respect to the fragmentation which gives a certain structural and chemical properties of that drug. To this end the mass spectra of a series of ''model drugs'' were measured at different fragmenter voltages from which breakdown diagrams were derived. The drugs were injected by FIA analysis and the fragmenter voltage was varied from 60 to 200 V (in steps of 5 V). The ion abundances of the corresponding mass spectra were transferred to Excel™, where the data were plotted as breakdown curves. The chromatographic conditions were as described above. The chromatographic separation was set up in a gradient elution and for that reason an average mobile phase of 50% ammonium formate–50% acetonitrile (with 0.05% formic acid added) at a flow of 0.7 ml/min was used, while the injection volume was decreased to 10 μl.

3. Results and discussion

Approximately 1000 spectra have been collected in positive mode to investigate the influence of several mobile phase conditions on the transport region CID of mass spectra of paracetamol and amitriptyline. Results are summarized in the following discussion.

3.1. Mass spectra at different organic modifier concentrations

Table 1 summarizes the ratios of the intensities of the peaks m/z 152=$(M+H)^+$ and m/z 110=$(M+H-CH_2O)^+$ from paracetamol and of the peaks and m/z 278=$(M+H)^+$ and m/z 233=$(M+H-HN(CH_3)_2)^+$ from amitriptyline at different concentrations of the organic modifiers methanol and acetonitrile in the aqueous buffer solutions of 0.1% acetic acid, 0.1% formic acid, 10 m*M* ammonium acetate, and 10 m*M* ammonium formate.

As may be noted from Table 1, changes in the concentration of the organic modifiers do not cause a dramatic variation in the peak intensity ratios.

However, the variation in intensity of the peak at m/z 152 from paracetamol turned out to be large compared with that of the peak at m/z 278 from amitriptyline. That is, a significant increase in intensity of the peak due to protonated paracetamol is observed at higher organic modifier concentrations (until 50%) which at even higher organic modifier concentrations decreases again. It can be concluded that the optimal organic modifier content is between 25 and 75%. There is also a significant difference between the two aqueous solutions of 0.1% acetic acid and 0.1% formic acid observed. The latter

Table 1
Absolute intensity of the protonated molecule from paracetamol (m/z 152) and amitriptyline (m/z 233) and the ratio between this peak and the second most intense peak, measured at different concentrations of the organic modifiers methanol and acetonitrile in buffer solutions (The fragmentor voltage setting for the presented data was 120 V)

	Paracetamol m/z 152						Amitriptyline m/z 278					
	Intensity	152/110	Intensity	152/110	Intensity	152/110	Intensity	278/233	Intensity	278/233	Intensity	278/233
Methanol (vol%)	25		50		75		25		50		75	
0.1% Acetic acid	42,664	0.27	35,114	0.28	20,961	0.29	913,000	1.92	1,020,000	2.17	1,080,000	2.50
0.1% Formic acid	76,344	0.26	76,358	0.30	57,477	0.30	1,280,000	1.67	1,320,000	1.59	1,470,000	1.67
10 m*M* Ammonium acetate	80,387	0.36	95,406	0.37	160,090	0.41	1,000,000	2.44	1,180,000	2.33	1,450,000	2.17
10 m*M* Ammonium formate	174,436	0.36	205,134	0.37	227,052	0.38	1,220,000	2.27	1,380,000	2.04	1,490,000	2.04
Acetonitril (vol%)	25		50		75		25		50		75	
0.1% Acetic acid	48,608	0.31	34,145	0.32	14,029	0.33	1,110,000	1.96	1,150,000	2.70	1,190,000	2.50
0.1% Formic acid	91,817	0.35	83,983	0.31	75,701	0.30	1,250,000	1.61	1,400,000	1.64	1,250,000	1.85
10 m*M* Ammonium acetate	71,525	0.34	87,924	0.37	117,737	0.48	1,000,000	1.92	1,200,000	2.27	1,570,000	2.27
10 m*M* Ammonium formate	164,026	0.41	174,174	0.49	181,142	0.40	1,270,000	2.27	1,410,000	2.17	1,570,000	2.17

showing a higher intensity of the protonated paracetamol (m/z 152) and amitriptyline (m/z 278).

Similar observations are made as described above when aqueous solution of 10 mM ammonium acetate or 10 mM ammonium formate are used. The intensity of the peak m/z 152 due to protonated paracetamol increases again much more than that of the peak m/z 278 due to protonated amitriptyline upon increase of the organic modifier concentration.

An explanation for this observation is given by the underlying complex relationship of the chemical nature of the compounds in solution given by Kebarle et al. [34] which describes, how analytes are transferred from solution to the gas phase.

The differences in behavior between paracetamol and amitriptyline find their origin in the difference of volatility between these two drugs.

3.2. Mass spectra at different buffer molarity and pH

Table 2 gives the ratios of the intensities of the peaks m/z 152=$(M+H)^+$ and m/z 110 $(M+H-CH_2CO)^+$ from paracetamol and of the peaks m/z 278=$(M+H)^+$ and m/z 233=$(M+H-HN(CH_3)_2)^+$ from amitriptyline at different concentrations of the organic modifier methanol and different pH and buffer molarities.

The ratios appear not to change notably at different organic modifier concentrations, but the intensities of the $(M+H)^+$ peaks decrease at lower pH because of an increased fragmentation of the $(M+H)^+$ ions.

Between the 10 mM and 100 mM buffer solutions there is a significant difference in the intensity of the protonated molecule signal from paracetamol (m/z 152) and amitriptyline (m/z 278). A possible explanation is ion suppression. This occurs when the vaporizing droplets in the spray chamber contains a high concentration of salt or other compounds [34,43].

3.3. Influence of the flow speed on the AP–ESI mass spectra

It is known that in electrospray the flow-rate is low. When the flow-rate is increased for a given analyte concentration, the sample ion signal does not increase. That is, in terms of analyte concentration the sensitivity remains constant, but in terms of mass flow the sensitivity drops when the flow-rate of the analyte solution is increased.

The apparent concentration sensitivity of ESI can be attributed to a decrease in droplet charging efficiency and a shift toward larger diameters in the droplet size distribution if the liquid flow-rate is increased. Both effects result in a lower ionization efficiency because of the reduced efficiency of the release of ions from the droplets. However, the introduction of more analyte molecules per unit time into the interface approximately compensates for this reduced efficiency, so that the signal level at the detector remains practically constant. This is confirmed by measurements done by the authors.

3.4. Inter-instrumental comparison of mass spectra

The inter-instrumental comparison of mass spectra can be divided in two parts. First a comparison is made between the mass spectra obtained with the two instruments used in the present study.

Table 3 shows the peak intensity ratios m/z 152/110 for paracetamol and m/z 278/233 for amitriptyline at different concentrations of the organic modifier acetonitrile as obtained with use of two different instruments.

The ratios appear not to be dramatically different for the two instruments and the same holds for the complete mass spectra.

Similar results have been obtained for seven other drugs in the test experiments. The observed differences in peak intensities may well be due to differences in the adjustment of the electrospray needle and the electronics in the two instruments. These differences appear not to influence the quality of library searching in negative sense as will be shown below.

The second inter-laboratory comparison of mass spectral data is made with data reported by Weinmann et al. [32]. Instruments of different vendors have different instrument settings to induce the fragmentation process which may hamper an inter-instrumental comparison of mass spectra. These difficulties may be circumvented by use of breakdown curves for the comparison. As an example, the breakdown curves for haloperidol, obtained with the

Table 2
Absolute intensity of the protonated molecule from paracetamol (m/z 152) and amitriptyline (m/z 278) and the ratio between this peak and the second most intense peak, measured at different mobile phase conditions, that is buffer molarity and pH (The fragmentor voltage setting for the presented data was 120 V)

	Paracetamol m/z 152						Amitriptyline m/z 278					
Methanol (vol%):	25		50		75		25		50		75	
	Intensity	152/110	Intensity	152/110	Intensity	152/110	Intensity	278/233	Intensity	278/233	Intensity	278/233
0.05% Acetic acid	58,757	0.64	37,716	0.65	52,196	0.64	1,850,000	1.64	1,580,000	2.08	1,810,000	1.82
0.1% Acetic acid	42,664	0.27	35,114	0.28	20,961	0.29	913,000	1.92	1,020,000	2.17	1,080,000	2.50
0.001% Formic acid	99,689	0.45	71,050	0.45	37,353	0.39	1,490,000	2.56	1,530,000	2.78	1,530,000	2.86
0.01% Formic acid	96,073	0.35	78,705	0.36	59,655	0.41	1,400,000	1.64	1,500,000	1.79	1,540,000	1.96
0.1% Formic acid	76,344	0.26	76,358	0.30	57,447	0.30	1,280,000	1.67	1,320,000	1.59	1,470,000	1.67
10 m*M* Ammonium acetate	80,387	0.36	95,406	0.37	160,090	0.41	1,220,000	2.44	1,380,000	2.33	1,490,000	2.17
100 m*M* Ammonium acetate	91,477	0.23	14,354	0.21	17,438	0.25	287,424	2.08	461,376	2.08	552,054	2.04
10 m*M* Ammonium formate	174,436	0.36	205,134	0.37	227,052	0.38	1,000,000	2.27	1,180,000	2.04	1,450,000	2.04
100 m*M* Ammonium formate	46,692	0.24	50,077	0.23	59,054	0.23	515,958	1.75	601,984	1.75	685,272	1.69

Table 3
Peak intensities ratios m/z 152/110 for paracetamol and m/z 278/233 for amitriptyline at different instruments (fragmentor setting 120 V)

	Paracetamol m/z 152/110			Amitriptyline ratio m/z 278/233		
Acetonitril (vol%)	25	50	75	25	50	75
G1946A instrument	0.41	0.43	0.40	2.33	2.17	2.17
G1946B instrument	0.54	0.60	0.66	1.82	2.94	1.79

A 10-mM ammonium formate solution was used as aqueous buffer. The G1946A instrument was located in the hospital pharmacy laboratory in Amsterdam, The Netherlands and the G1946B instrument in the Agilent application laboratory in Amstelveen, The Netherlands (see Section 2).

G1946A and G1946B instruments have been compared with those reported by Weinmann et al. [32] as obtained with different types of PE/Sciex™ instruments (Fig. 1).

A similar result gave the comparison of the breakdown curve of diazepam obtained on the G1946A instrument with that reported by Weinmann et al. [32] (Fig. 2).

These results indicates that ''exchangeable libraries of ESI–CID spectra'' could be generated successfully.

3.5. Optimization of the fragmenter voltage for a mass spectra library generation

A mass spectral library search with a good fit is only possible if the ionization conditions used for the sample are similar to those used for setting up the library. For GC–EI spectra of most compounds the ionization efficiency curve is not changing anymore at an electron energy above 40–50 eV. Consequently the standard electron ionization of 70 eV is no longer a parameter, which varies the mass spectrum. This is different for CID which by energetic collisions in the transport zone can be achieved by increasing the skimmer, nozzle, cone, capillary and orifice voltage, respectively, depending on the construction of the source. Increasing the collision energy by increasing the fragmenter voltage gives at a certain voltage optimal information. If this voltage is further increased, the analytes will be completely fragmented. Optimization and standardization of this method of CID by energetic collisions are very difficult due to differences in the design of the different types of

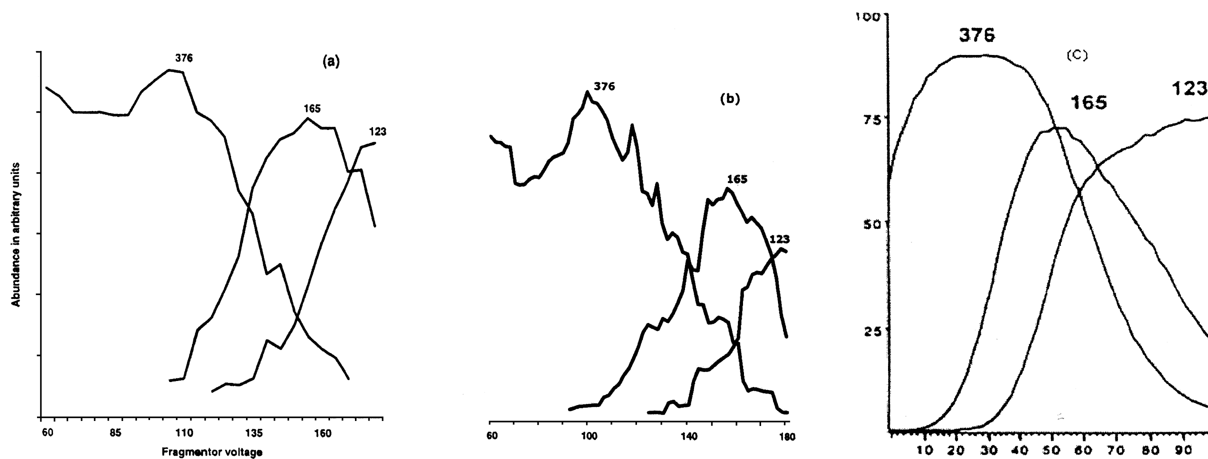

Fig. 1. Breakdown curve of haloperidol (concentration 0.05 mg/l in 50% 0.05% TFA and 50% acetonitrile) obtained in steps of 5 V increase of the fragmenter voltage on (a) the G1946A and (b) the G1946B instrument. For comparison the breakdown curve (c) as reported by Weinmann et al. [32].

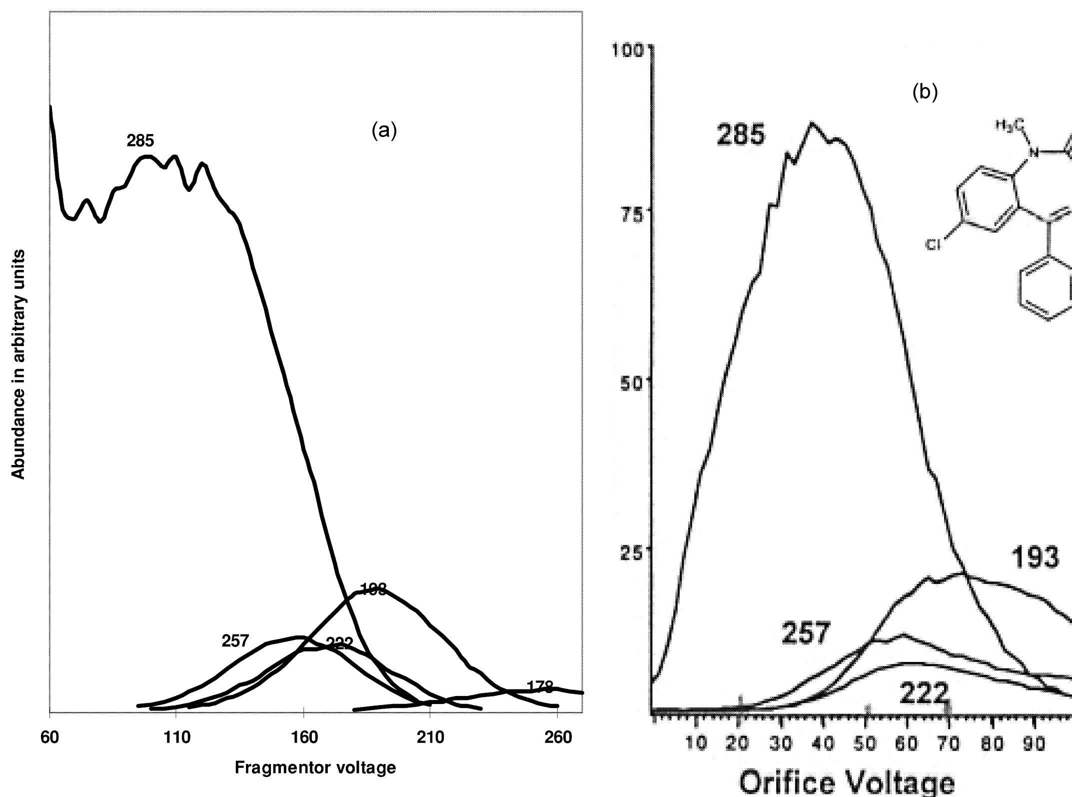

Fig. 2. Breakdown curve of diazepam obtained with (a) a G 1946A instrument (left) and (b) a PE/Sciex instrument [32].

instruments. Even a comparison between instruments of the same brand may show small differences (i.e. pressure in the CID region), which may influence the appearance of the resulting mass spectra. Bogusz et al. [13] cited in their conclusion, where they tested the inter- and intralaboratory comparability of CID mass spectra: ‘‘the entire material gathered in this study is not a mere collection of irreproducible results.’’

When optimizing the fragmenter voltage, both optimized fragmentation and standardized fragmentation have to be taken in account. For finding the optimized fragmentation the measured breakdown curves have been used. The breakdown curves of most drugs show a specific contribution of relevant ions to the fragmentation pattern. A few examples of breakdown curves are presented in Fig. 3.

In general more energy is required for larger ionized molecules to induce fragmentation as internal energy obtained from collision is partitioned over a larger number of vibrational degrees of freedom. This is confirmed by the breakdown curves for paracetamol and amphetamine which show that at higher fragmenter voltages the ionized molecules are completely fragmented. This makes the choice for an optimal fragmenter voltage during acquisition of library spectra and for spectra from unknown samples more difficult. For larger ionized molecules also more kinetic energy is necessary to transport them effectively through the CID region. This means that the fragmenter voltage must also be optimized for an optimal transmission of the masses of interest which can be achieved with use of the available ‘‘autotune’’ algorithm. For the latter the qualitative choice, based upon the measured breakdown curves, has been made to increase for every mass the fragmenter voltage with 25 V.

During data acquisition, each scan is dynamically ramped depending on the fragmenter voltage based on mass, for which the desired ramp is entered in a mass/fragmenter table (Fig. 4).

The application of the fragmenter ramp has two

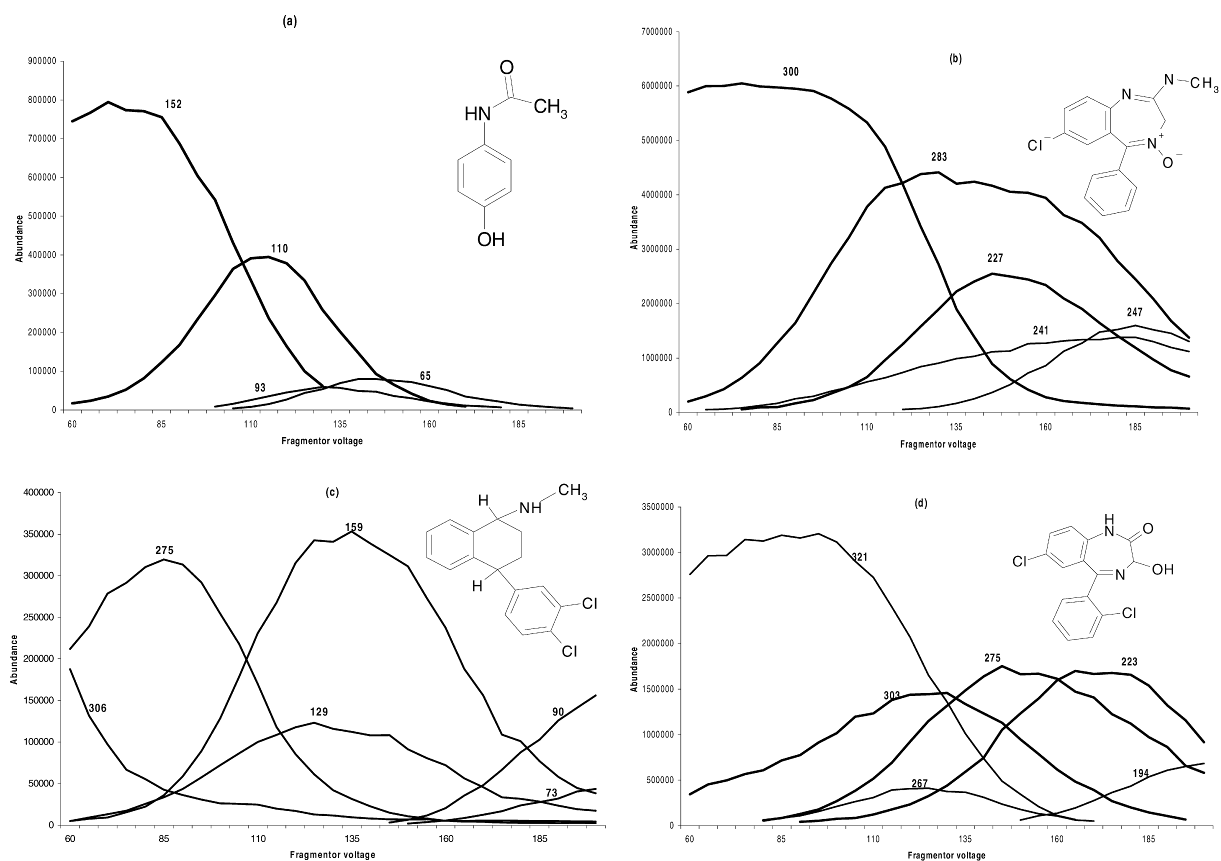

Fig. 3. Breakdown curves of (a) paracetamol (an analgesic), (b) chlorodiazepoxide (a benzodiazepine), (c) sertraline (an antidepressant) and (d) lorazepam (a benzodiazepine). Mobile phase conditions: 10 m*M* ammonium formate–acetonitrile (0.05% formic acid added) (50:50, v/v). The applied flow is 0.7 ml/min. and the injection volume 10 μl. Data was obtained with G1946A instrument.

advantages. First, larger ionized molecules receive more kinetic energy which improves their transmission and second, the fragmentation efficiency will increase in the CID transport region. CID in the ESI transport region has a high efficiency (80–95%) [33] because fragmentation occurs in the relatively high pressure supersonic expansion between the capillary nozzle and the skimmer nozzle. During the autotune the fragmenter voltages for the analyzer are optimized for the particular instrument used.

3.6. Optimization of a generic analytical method for the analysis of STA

Even with highly sophisticated instrumentation, such as HPLC–AP–ESI–MS in the present study, a substance can not be found when it is not extracted.

In studying the literature and highlighting the extraction procedures, it is remarkable that there are as many approaches to the extraction of drugs from body fluids as there are articles about this topic [37–42]. In STA the substance present is not a priori known. In such an undirected search the extraction procedure cannot be aimed at a given substance, but must be general, in which a compromise must be reached such that the drugs of interest are extracted with a yield as high as possible and that the interfering substances from the plasma are removed. In this study the choice has been made for the traditional liquid–liquid extraction (LLE).

Although LLE has proven to be suitable in a considerable number of cases, this technique has

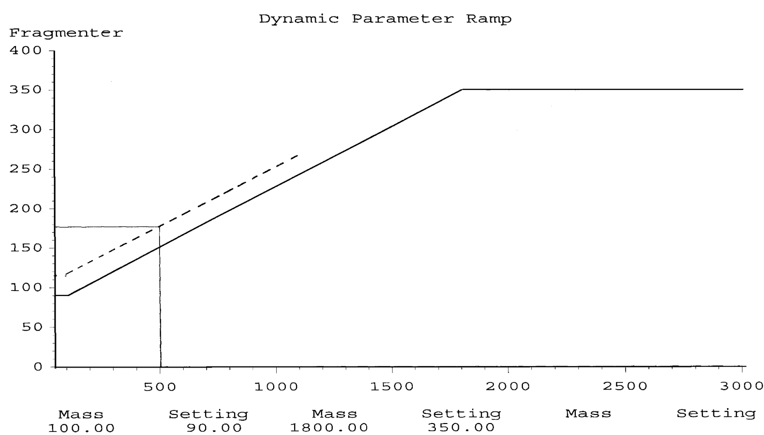

Fig. 4. Fragmenter ramp obtained during the autotune of the mass analyzer calibration. The dotted line is the dynamic fragmenter voltage ramp during the scan.

some disadvantages, such as matrix interference, emulsion formation and use of large volumes of hazardous solvents [38].

In recent years, increasingly more STA extraction procedures are based on solid-phase extraction (SPE). Many different column materials are available and for the future the SPE technique looks promising. In this study the LLE was preferred over SPE.

HPLC is a very valuable method for STA because of its high separation power and its applicability to a large number of compounds, including non-volatile and thermolabile compounds. Since the recent introduction of highly reproducible column material, this method, originally criticized because of a poor reproducibility of retention times (t_R) possesses now a good selectivity due to reproducible t_R values. The highly reproducible column material is based upon a stable bond packing which is achieved by chemically bonding a sterically protected C_{18} stationary phase to a porous silica microsphere. This column material, used in this study, has been designed to reduce or eliminate the strong adsorption of basic compounds, being the majority of the molecules studied. Another reason for this column choice is the extreme low pH, which this column type allows. Without any addition of additives, which would increase the ESI–MS background, the peaks are symmetrical.

Table 4 lists the tested drugs and their specific retention times.

The chromatogram of a standard mixture of drugs is given in Fig. 5. Not all the drugs are well separated, but this is for the STA not really necessary.

3.7. Development of a mass spectral drug library

For setting up a mass spectral library of drugs of clinical and forensic interest (e.g. drugs of abuse, benzodiazepines, analgesic, antidepressants, etc.) blank plasmas have been spiked with the drugs at the toxicological level concentration. Subsequently the plasmas have been extracted according to the basic liquid extraction procedure.

This approach has been chosen to obtain "more realistic" mass spectra. That is, drugs may undergo degradation or conjugation during the extraction process, which will affect their mass spectra. This technical approach is not optimized for drugs, which are metabolized in vivo. Such an extension of the library can only be made if samples of patients are available. A library search of entries has been tested with a series of FIA injections of methanol diluted standards. The results are shown in Table 5.

Tables 5–7 show three numbers for each spectrum. The first column (M) shows a match factor for the unknown and the library spectrum (direct match). The second column (R) gives the match factor for the unknown and the library spectrum ignoring any peaks in the unknown that are not in the library

Table 4
Molecular weight, retention times (t_R), toxic concentration, and the used liquid–liquid extraction (LLE) of the tested compounds spiked in blank human plasma or patient plasmas

Compound	Molecular mass	Toxicological concentration (mg/l)	t_R	Extraction[b]
Morphine	285.3	0.2	3.6	Basic
Sotalol	272.4	5.0	6.7	Basic
Paracetamol	151.2	120	7.2	Basic/acid
Codeine	299.4	0.9	7.7	Basic
Olanzapine	312.4	0.04	7.9	Basic
Caffeine	194.1	20	8.3	Basic
Monodesethylamiodaron[c,d]	619.3	5	9.2	Basic
Amphetamine	135.2	0.2	9.6	Basic
Lamotrigine	256.1	4.5[a]	9.8	Basic
Phenytoin	252.3	20	11.0	Basic
Nevirapin	266.3	6.5[a]	11.3	Basic
Phenobarbital	232.2	60	11.8	Acid
Clozapine	327.0	0.8	12.0	Basic
Midazolam	325.8	0.25	12.6	Basic/acid
Flurazepam	387.0	0.15	12.8	Basic
Paroxetine	329.0	75[a]	13.1	Basic
Promethazine	284.4	1.0	13.1	Basic
Flecainide	414.4	1.0	13.1	Basic
Haloperidol	375.9	0.05	13.4	Basic
Promazine (IS)	320.9	–	13.5	Basic
Oxazepam	286.7	3.0	13.8	Basic
Phenprocoumon	280.3	5	13.8	Acid
Desmethylclobazam	286.7	4.0[a]	14.1	Basic
Lorazepam	321.2	0.3	14.1	Basic
Naproxen	230.3	75	14.4	Basic/acid
Amitriptyline	313.9	0.4	14.5	Basic
Zoplicon	388.8	0.15	14.5	Basic
Sertraline	306.2	0.3	14.8	Basic
Zuclopenthixol	474.0	0.3	15.0	Basic
Triazolam	324.0	4	15.2	Basic
Flunitrazepam	313.3	0.05	15.2	Basic
Desmethyldiazepam	270.7	1.5	15.3	Basic
Saquinavir[c]	766.0	1.4[a]	15.3	Basic
Pimozide	461.6	0.005	15.3	Basic
Clomipramine	314.9	0.4	15.4	Basic
Clobazam	300.7	0.4	15.7	Basic
Valproic acid	144.1	150	16.1	Acid
Chlorodiazepoxide	299.8	3.5	17.1	Basic
Diazepam	284.8	1.5	17.7	Basic
Ritonavir[c]	720.7	9	19.1	Basic
Ibuprofen	206.0	100	19.7	Acid

[a] For some compounds, no toxicological concentration is described. For these compounds the upper therapeutic dose has been used.
[b] This column gives how the compounds to be identified have been extracted. Mass spectrometry has been performed in the negative ion mode for compounds extracted ''acid''. Some compounds have been detected in both basic and acid extracts.
[c] Some compounds have molecular masses beyond the scan range of m/z 500, but some specific fragment ions could be detected.
[d] Desethylamiodaron has not been detected.

spectrum (reverse search). A perfect match results in a value of 1000; spectra with no peaks in common result in a value of 0. As a general guide, 900 or higher is an excellent match; 800–900 is a good match; 700–800 a fair match. Less than 600 is a very poor match. However, unknown spectra with many

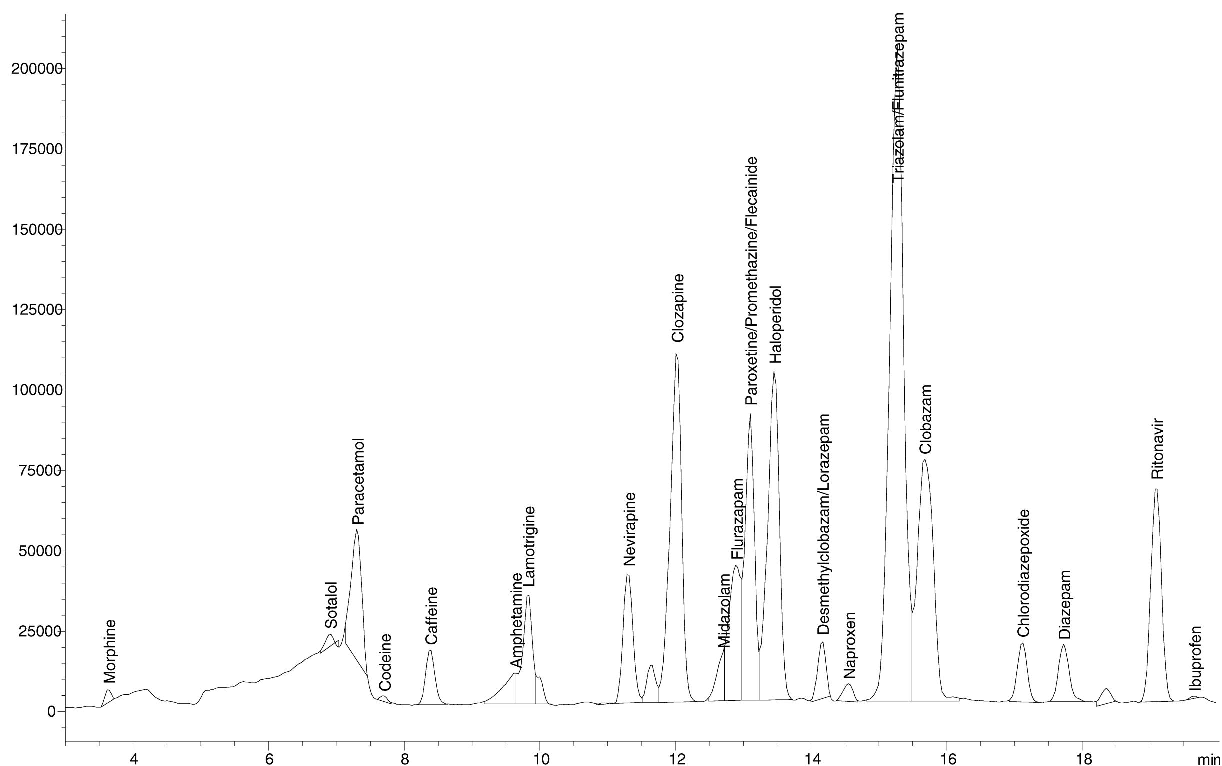

Fig. 5. Chromatogram of 25 drugs. The concentration of the components is at the toxicological level. Some components are overlapping (although ion suppression might have occurred, all components of the co-eluting peaks at the retention times of 13.0, 14.1 and 15.2 min, could be identified). A Zorbax™ stable bond C_{18} column (25 cm, 4.6 mm I.D. and 5 μm particles) is used. Flow rate: 0.7 ml/min. The gradient started with 90% 10 m*M* ammonium formate at pH 2.9 and 10% acetonitrile (0.05% formic acid added). Linear increment acetonitrile concentration from time 0 to 10 min. From 10 to 24 min the concentration of acetonitrile is increased to 80% and from 24 to 26 min to 100%. Isocratic at 100% until the stop time at 30 min. Equilibration time 7 min. Oven temperature 40°C. Injection volume 25 μl. The MS conditions are described in the instrumental section.

peaks will tend to yield lower match factors than otherwise similar spectra with fewer peaks. The third column shows the probability (P), which is derived assuming that the compound is in the database and which relies only on the differences between adjacent hits in the hit list [35,36].

All drugs, except phenobarbital, are correctly assigned. Some match values are low due to the sometimes very low toxicological concentrations. Table 5 summarizes also the library search results for two other series of spiked plasma samples acquired with two different instruments. The matching results obtained with the G1946A instrument are better than those of the G1946B instrument simply because for the identification of the spectra from both instruments only the library collected with the G1946A instrument has been used.

At any rate, the match values show very promising results, but real samples of patients are the only way to test the robustness of the developed method and library.

To this end a series of samples of patients with known medication has been collected and the mass spectra measured. Moreover, the drugs known because of the medication have first been spiked and their spectra collected and stored in/added to the library as described above. The results obtained have been summarized in Table 6.

Table 5
Library search of standard solutions (toxicological concentration) dissolved in methanol and spiked to blank plasma (N.D. means not detected)

Compound	In methanol[a]			Spiked in blank plasma					
				Measured with G1946A[b]			Measured with G1946B[c]		
	M	*R*	*P*	*M*	*R*	*P*	*M*	*R*	*P*
Phenobarbital	N.D	N.D	N.D	N.D.	N.D	N.D	N.D.	N.D	N.D
Morphine	965	982	58.6	959	999	82.7	893	974	87.2
Paracetamol	767	938	99.0	935	996	98.8	264	804	28.3
Codeine	575	989	53.2	971	999	62.0	952	999	62.0
Olanzapine	999	999	98.8	999	999	98.8	625	706	80.9
Amphetamine	251	956	67.0	986	999	98.7	N.D	N.D	N.D
Midazolam	953	964	64.2	937	990	56.8	937	946	48.6
Flurazepam	986	999	97.0	931	947	99.0	748	952	94.3
Haloperidol	472	929	69.8	953	991	52.0	949	978	88.0
Lorazepam	893	923	97.0	867	964	98.9	705	944	79.7
Amitriptyline	882	954	83.3	859	998	94.2	844	865	94.2
Naproxen	729	954	99.0	740	905	98.5	297	837	9.3
Zoplicon	876	970	98.4	934	999	99.0	464	792	90.6
Sertraline	945	982	97.6	860	965	76.4	874	903	83.6
Flunitrazepam	969	978	79.8	999	999	98.8	478	894	54.3
Pimozide	807	851	98.2	940	991	98.5	552	937	97.3
Chlorodiazepoxide	835	919	97.0	953	999	98.8	720	733	98.7
Diazepam	969	983	81.3	930	935	53.7	960	973	72.1

[a] The mass spectra in the library have been collected as described in the text, that is they have been obtained from the methanol solution with the G 1946A instrument and then stored in the library.
[b] Spectra obtained form the G1946A instrument and identified with use of the library collected with this instrument.
[c] Spectra obtained from the G1946B instrument and identified with use of the library collected with the G1946A instrument.

In some cases the concentration of the drugs in the ''patient'' samples turned out to be very low (or there was not enough sample available); in these cases a positive identification has been achieved by comparison with a blank plasma. Such identification of ''unknown drugs'' appeared to become easier, if additionally a comparative overlay between the ''patient'' chromatogram and the blank chromatogram, as monitored by the mass spectrometer, is made. An example is given in Fig. 6.

Basic, and neutral drugs are best recovered by basic liquid–liquid extraction (LLE). Acid compounds (i.e. ibuprofen, naproxen, phenobarbital), however, show low recoveries or are not extracted at all using this procedure. Therefore, the method of acid LLE has been applied to and tested on acidic drugs with a low pK_a. Although in this case the pH of the used mobile phase is very low, the mass spectrometric measurement has been performed in the negative ion mode.

Fig. 7 shown an example of an acid LLE chromatogram as monitored by the mass spectrometer.

Table 7 presents the library search results for a series of spiked acidic drugs.

4. Summarizing conclusions

The potential of HPLC–AP–ESI mass spectrometry as an identification tool for drugs in biosamples, relevant to forensic and clinical toxicology, has successfully been demonstrated in this study.

Changes in the composition of the mobile phase constituents have been shown not to affect the reproducibility of mass spectral data to any relevant extent. Mass spectra obtained in the positive ESI mode have been observed to remain similar during the duration of this study over more than 8 months.

Table 6
Library search results of a series of spiked plasmas and a series of samples of patients (The concentration of the samples of patients is unknown)

Compound	Spiked blank pool plasma				"Patient" sample			
	M	*R*	*P*	t_R	*M*	*R*	*P*	t_R
Monodesethylamiodaron	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Valproic acid	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Phenprocoumon	N.D.	N.D.	N.D.	N.D.	N.D.[c]	N.D.	N.D.	N.D.
Sotalol	928	968	98.9	6.7	841	905	96.5	6.7
Caffeine	921	981	95.4	8.3	899[c]	990	97.9	8.3
Lamotrigine	989	992	98.3	9.8	963[a,c]	983	98.6	9.8
Phenytoin	935	997	23.2	11.0	859	989	53.1	11.0
Nevirapine	952	993	96.2	11.3	N.A.	N.A.	N.A.	N.A.
Clozapine	967	991	98.8	12.0	893	985	98.0	12.0
Paroxetine	886	942	98.3	13.1	563[a,c]	925	98.2	13.1
Promethazine	719	747	98.9	13.1	N.D.	N.D.	N.D.	N.D.
Flecainide	960	988	98.9	13.1	934[c]	965	98.8	13.1
Promazine (ISTD)	899	996	96.5	13.5	899	998	81.9	13.5
Desmethylclobazam	877	917	99.0	14.1	876	923	99.0	14.1
Zuclopenthixol	927	990	98.5	15.0	565[c]	919	19.9	15.1
Triazolam	956	985	98.7	15.2	870[b]	933	98.5	15.2
Desmethyldiazepam	902	993	98.7	15.3	882	921	98.8	15.2
Saquinavir	836	980	98.2	15.3	755	941	98.8	15.4
Clomipramine	811	915	99.0	15.4	772	897	99.0	15.5
Clobazam	969	987	98.8	15.7	N.A.	N.A.	N.A.	N.A.
Ritonavir	938	994	98.7	19.1	861	990	98.9	19.1
Ibuprofen	369	517	78.6	19.5	N.D.	N.D.	N.D.	N.D.

N.D. is not detected; N.A. is not available as "patient" sample.
[a] Hemolytic plasma.
[b] Icteric plasma.
[c] Less plasma than the amount needed (1 ml) for normal extraction.

This also holds for the inter-instrumental comparison of mass spectra, which enables to generate an applicable database of ESI mass spectra.

Although the mobile phase conditions can be neglected, standardization of the fragmenter voltages is required to obtain reproducible and inter-instrument comparable mass spectra. This has been achieved by estimating the optimum voltage for fragmentation from breakdown curves, which have been measured for a number of standard drugs on different instruments.

Table 7
Library search results of a series of spiked acidic drugs[a]

Compound	Spiked sample			
	M	*R*	*P*	t_R
Phenobarbital	835	949	99.0	11.8
Phenprocoumon	951	999	98.8	18.4
Paracetamol	938	972	98.8	7.2
Valproic acid	818	856	99.0	16.1
Ibuprofen	859	981	98.8	19.7
Naproxen	880	995	98.8	14.4
Monodesethylamiodaron	N.D	N.D	N.D	–

[a] Apex mass spectra have been compared with library entries without background correction. All drugs, except monodesethylamiodaron (mass>500, no fragments identified), are correctly assigned.

For setting up a library of CID mass spectra, spiked blank plasmas have been extracted with use of basic LLE followed by separation via HPLC. Mass spectra of in total about 40 different drugs have been recorded and stored, for which the fragmentation was induced by the use of a ramped fragmenter voltage.

Several tests have been performed to check the reliability of the generated CID–ESI drug library. To this end the tested drugs were primarily extracted with basic LLE and measured in the positive ESI mode. Plasmas of acidic drugs were extracted with

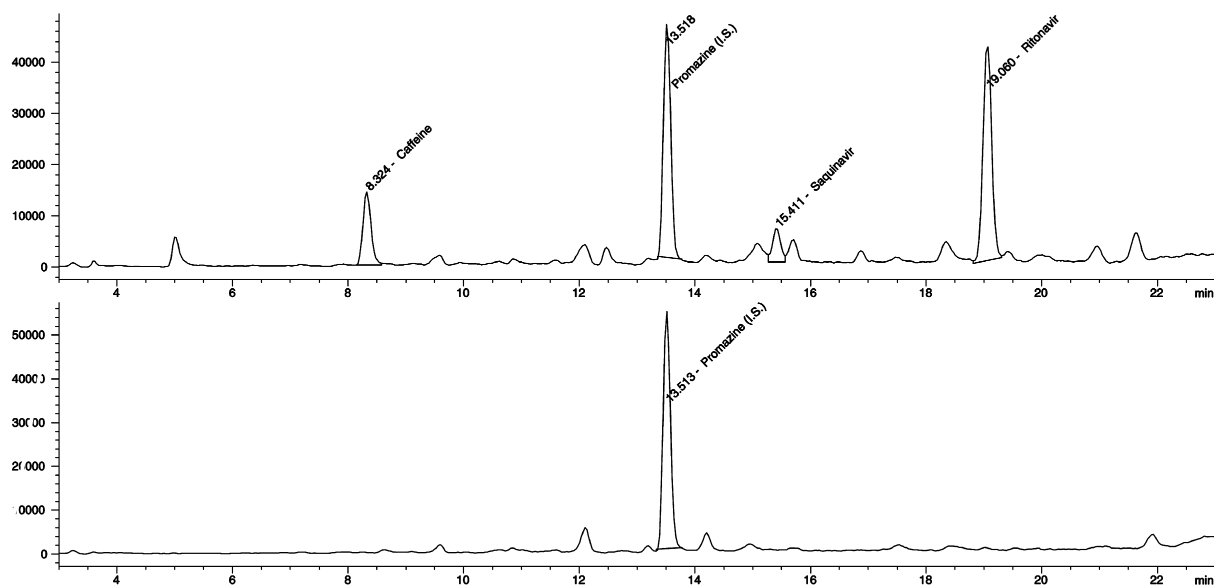

Fig. 6. Chromatogram of a ''patient'' sample obtained in the positive ion mode (top). The drug saquinavir has been identified by comparison with a chromatogram of a blank sample (bottom). Promazine has been added to the extraction buffer as internal standard. Besides the drug saquinavir, also the drugs ritonavir and caffeine have been identified in this way.

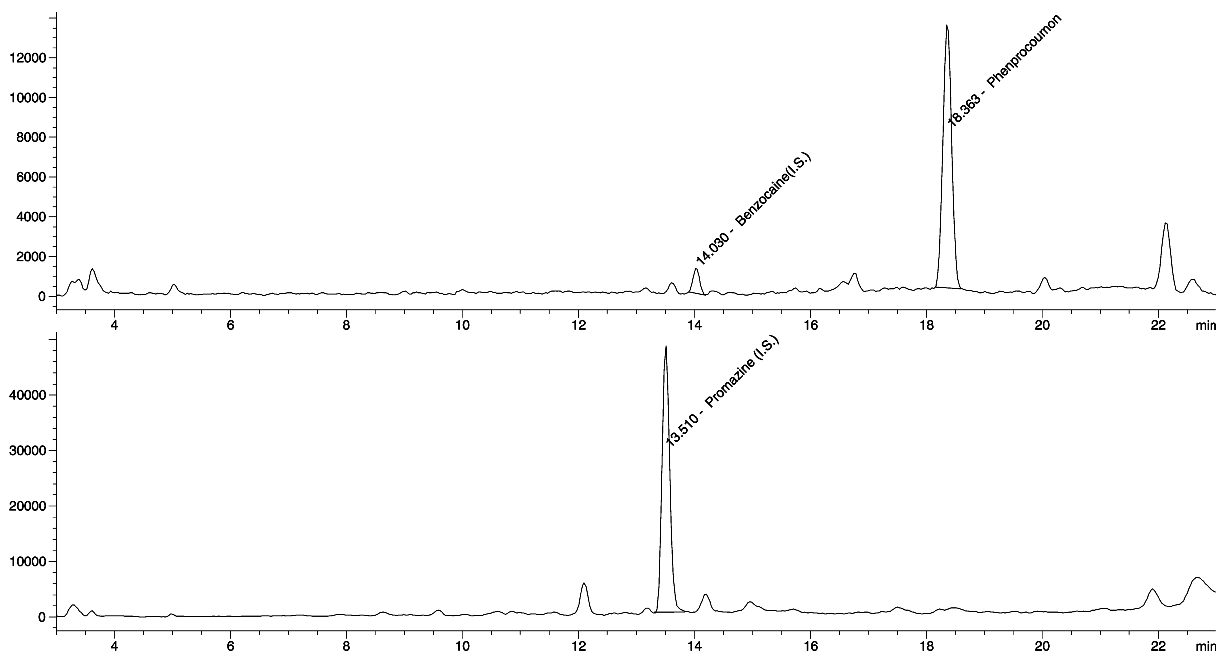

Fig. 7. Top: chromatogram of an acid (LL) extracted blank human plasma spiked with phenprocoumon as monitored by detection of $(M-H)^-$ in the negative ion mode. Bottom: same plasma extracted with basic LLE and monitored in the positive ion mode. To both samples an internal standard has been added for control of the extraction.

acid LLE and their mass spectral data obtained in the negative ESI mode.

A number of series blank plasma samples randomly spiked with a drug and a series of samples from patients with known medication have given in this way an average library search result of more than 95% positive identification.

It is clear that for a successful drug library search the procedure for unknown drug screening should contain a combination of both an acidic and a basic extraction, the latter to be analyzed in the positive and the former in the negative ion mode.

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