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The Application of Liquid Chromatography and Capillary Zone Electrophoresis Combined with Atmospheric Pressure Ionisation Mass Spectrometry for the Analysis of Pharmaceutical Compounds in Biological Fluids

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Abstract. HPLC coupled to atmospheric pressure ionisation mass spectrometry has almost replaced HPLC assays with UV, fluorescence, or electrochemical detection, due to its enhanced speed, sensitivity, and selectivity, especially when tandem-MS is used. To increase the speed and sensitivity of the drug assays further, high-speed HPLC, multi-component analysis, and μHPLC are used on a routine basis. Sample preparation is recognized as an important issue in bioanalytics. The use of a 96-well plate format with automated liquid-handling systems, off-line and on-line solid-phase extraction or automated liquid-liquid extraction allows to cope with the high sample throughput enabled by LC-MS. Although LC-MS/MS represents the highest standard with respect to sensitivity and selectivity, LC-MS, as a less expensive alternative, is useful in many stages of drug discovery and development. CE-MS and CEC-MS appear to be an attractive alternative to HPLC-MS with respect to separation power, but both are a challenge in application and only seldomly used for the quantification of new drug candidates in biological fluids.

1. Introduction

Atmospheric pressure ionisation (API), a soft ionisation method which allows the transfer of polar and thermolabile compounds from the liquid to the gas phase, has brought liquid chromatography combined with tandem mass-spectrometric detection (LC-MS/MS) to the forefront of analytical techniques encountered in the pharmaceutical industry to support drug discovery and drug development [1–4].

During discovery and development of new drug candidates, there is a fundamental need to determine accurately and precisely the concentration of the drug and its relevant metabolites in various biological matrices. Over the last decades, mainly liquid chromatography combined with UV, fluorescence, or electrochemical detection was applied successfully to the quantification of the target compounds in plasma, urine, or tissue samples originating from pharmacokinetic and toxicological studies. At that time, gas chromatography combined with mass-spectrometric detection (GC-MS) was already a well-established and robust technique. Unfortunately, due to the thermolabile nature of many pharmaceutical compounds, and in particular their metabolites which are often more polar than the drug itself, GC-MS without derivatisation could be used only in a few cases. When working with biological samples, GC-MS requires always an extensive sample clean-up.

A mass spectrometer can be considered as a universal detector, assuming that the analytes can be ionized and sampled into the mass analyzer without degradation. The combination of a separation technique, such as gas or liquid chromatography, with mass spectrometry was highly desirable due to enhanced sensitivity and selectivity of the detector. While GC-MS has been a well-established techniques for more than 25 years, LC-MS did not catch on challenging technical limitations such as the transfer of the analyte from the liquid phase to the low-density gas phase. On the other hand, there is a basic incompatibility between large amounts of liquid coming out of a LC column and a detection system that works under vacuum.

With API techniques, the LC mobile phase is nebulised to form a spray of very small droplets by means of heat, electric, and pneumatic forces [5]. Ions escaping from these droplets are sampled into the vacuum by electric forces, while the mobile phase remains outside the mass ana-

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lysers. When appropriate solvents are used as mobile LC phases, *e.g.* water, acetonitrile or methanol and volatile buffers, LC-MS with API becomes a robust analytical tool.

There are two ways to generate ions at atmospheric pressure. With pure electrospray [6], the liquid is atomised with the help of a high electric field (3–5 kV) applied at the tip of a sprayer. Due to the strong electric field and redox processes, charge separation occurs in the *Taylor* cone formed on the sprayer tip, and positively charged droplets, in the case of positive-ion mass spectrometry, are emitted. Droplet size decreases by evaporation of solvent molecules leading to an increase in charge density. When charge density reaches a certain value, repulsion of the charges exceeds the *Rayleigh* limit of the droplet, and ions are emitted from the droplets. Pure electrospray is limited to a flow rate up to 10 µl/min, which is not compatible with common HPLC flow rates using 4-mm i.d. HPLC columns. The introduction of ion spray and, later, turbo ion spray, where the spray formation is assisted by a nebulising gas and heat, has lead to stable sprays for flow rates up to 2 ml/min.

While electrospray is a condensed-phase ionisation process which works best for ions in solution, atmospheric pressure chemical ionisation (APCI) is a chemical ionisation gas-phase process [7–11]. In a first step, electrons emitted by a corona discharge react with solvent molecules to produce the reagent gas. Then, in positive mode, the protons are transferred to the analyte by proton transfer. In APCI, the spray is formed with a nebulising gas (nitrogen or air) and heat. The response of the analyte is dependent on its gas-phase proton affinity. Both procedures are soft ionisation techniques and produce similar spectra either in positive or in negative mode with almost no fragmentation. API is particularly suitable for polar thermolabile compounds, which can be protonated or deprotonated either in the condensed phase or in the gas phase. The flow rates, which are practicable with modern instruments, are in the range of 0.005–1.0 ml/min for ion spray and 0.2–2 ml/min for APCI [12]. Combinations of API with all types of mass analyzers have been described [5]. However, triple-quadrupole instruments (TQ) are currently the most common mass analyzers used for quantitative LC-MS. This is due *i*) to historical reasons, because the first commercially available instruments were TQ, and *ii*) to the fact that MS/MS brings the required selectivity and compensates for the lower separation power of LC, particularly when short analysis times are desired, in comparison to GC.

Capillary zone electrophoresis (CZE) is another liquid separation technique where the analytes are separated by means of different ion mobilities originating from different charge-to-size ratios under a strong electric field. This technique is simple to use and inexpensive and can provide millions of plates for separation. While LC-MS is nowadays a well-established robust technique with known limitations, CZE-MS remains an attractive, but difficult methodology with many open questions [13][14]. However, CZE-MS offers an different selectivity compared to LC, and currently much research is carried out in this field. Another powerful separation technique called capillary electrochromatography (CEC) [15] is carried out with packed capillary columns in which the very small liquid flow rate is generated by electroosmosis; however, this techniques is still in its early stage.

The aim of this paper is to discuss status and applications of LC-MS and CE-MS for the determination of pharmaceutical compounds and their metabolites in biological fluids.

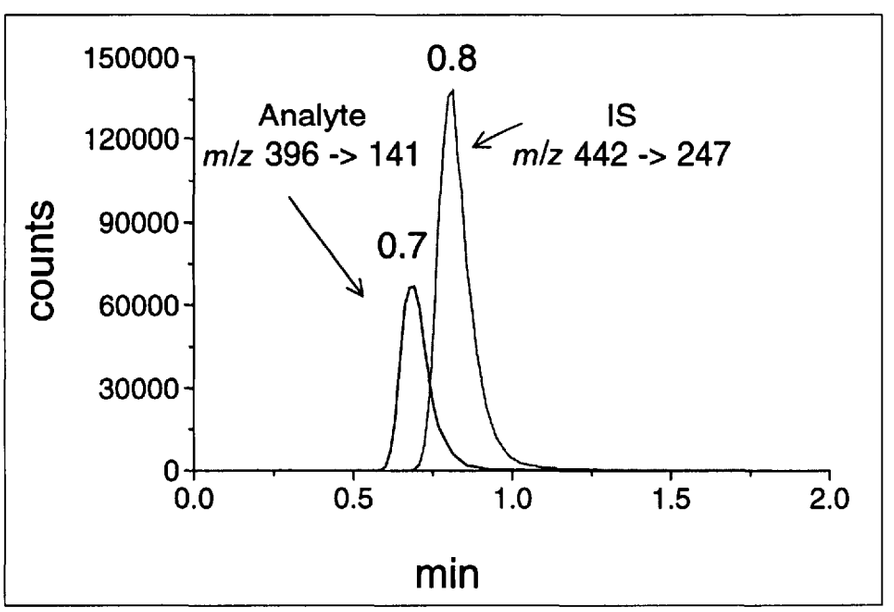

Fig. 1. *SRM Chromatograms for the analyte and its internal standard.* Rat-plasma sample containing 56 ng/ml of the analyte after oral administration of analyte.

2. High-Speed LC-MS/MS

The high potency of new drugs and aggressive time lines in the pharmaceutical industry call for more sensitivity, selectivity, and also higher throughput to analyse – below the nanogram-per-ml range – thousands of samples in a very short period of time.

Typical HPLC-UV runs are in the range of 10–20 min, allowing the analysis of about 36–72 samples within 12 h. In the case of LC-MS/MS, run times are in the range of 3–5 min for one or two analytes allowing an approximately three-fold increase of the sample number.

With API, the biological matrix can suppress severely or, in some cases, even enhance the ionisation of the analytes. Therefore, sample preparation remains an important step in the analytical procedure [16]. On-line analytics to support clinical trials or very fast response times demand high-sample-throughput methods. *Fig. 1* illustrates this with representative selected reaction monitoring (SRM) chromatograms of a rat plasma assay. The analyte co-elutes after 0.8 min with a structural analog used as internal standard. Due to the unique selectivity of tandem mass spectrometry in selected reaction monitoring (SRM) mode, baseline separation of the two peaks is not required. To achieve such short retention in the isocratic mode, the standard-bore analytical column (*CN HP Nova-Pak*) was only 20 mm long with an internal diameter of 4.6 mm. The HPLC mobile phase consisted of a mixture of acetonitrile/20 mM ammonium formate (50/50 *v/v*) with 1% formic acid, and was delivered at a flow rate of 0.8 ml/min. The analyte and the internal standard were isolated from rat plasma by liquid-liquid extraction with 1-chlorobutane/propan-2-ol. Mass spectrometric detection was performed in the SRM mode with positive-ion detection at a dwell time of 250 msec

using the APCI-heated nebuliser. The assay was linear from 10 to 10000 ng/ml using a 100 µl plasma aliquot, as illustrated in *Fig. 2*. The mean precision and the accuracy of the assay determined from QC samples was 3.3 and 99.9%, respectively. More than 300 samples could be analyzed within 12 h using this assay so that sample handling and data handling became the rate-limiting steps. A high level of automation is required to fully benefit from such approaches. When metabolites are quantified, special attention has to be given to the occurrence of various isomers which might generate similar mass spectra and, thus, falsify analytical results.

The further step of high-throughput methods toward high sample numbers was recently demonstrated by *Zweigenbaum et al.* [17]. The authors reported the analyses of six benzodiazepines in 1000 human urine samples within 12 h maintaining acceptable precision and accuracy. The analytes were chromatographed on a 2.1 mm i.d. × 15 mm long LC column packed with 3 µm particle-size material at a high flow rate of 1 ml/min. The elution of the six analytes was carried out isocratically within 30 s. The mass spectrometer used for this work was a TQ *API 3000* (*PE Sciex*, Concord, Ontario) with turbo ion spray in positive ion mode. Acquisition was performed in SRM mode. Modern TQ instruments are capable of very fast scanning without collision-cell carry-over, and dwell times in the SRM mode are possible in the 10-ms range. This is mandatory to record a sufficient number of points over the narrow chromatographic peaks. One device which causes many problems in quantitative LC-MS is the autosampler. These instruments are generally slow, have limited sample-number capacities, and are prone to carry-over. To achieve an injection every 38 s, the authors had to connect four autosamplers to an in-house-made switching system. They demonstrated the feasibility of this approach, but it is certainly not practicable for routine analysis at present. Innovative hardware development is mandatory in this field to really benefit from such approaches for routine work.

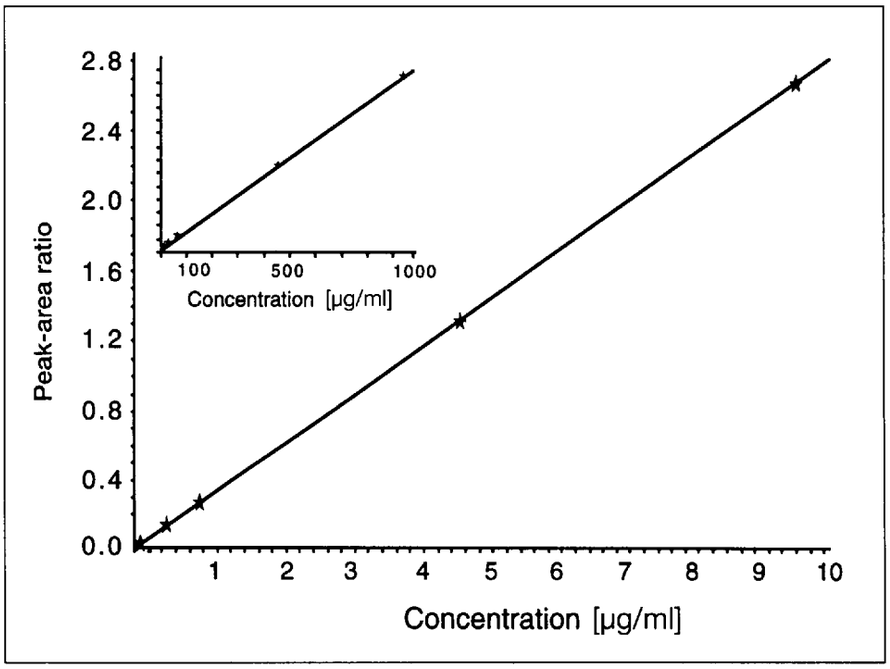

Fig. 2. *Calibration curve for the analyte from* Fig. 1, *from 10 to 10000 ng/ml*

3. Sample Preparation for LC-MS/MS

Quantitative results obtained by LC-MS to support pharmacokinetic (PK) studies can be affected by lack of sensitivity, selectivity, or specificity caused by *i*) ion suppression due to matrix effects, *ii*) interference from metabolites, *iii*) carry-over which may occur in the high-pressure collision cell, and *iv*) contamination of the deuterated internal standard (IS). Contrary to common perceptions a good sample preparation as well as the use of an adequate IS, preferably a stable isotopically labeled analog of the analyte, is an important key to success. Liquid-liquid extraction and solid-phase extraction (SPE) remain the most favored approaches for sample preparation. To be able to benefit fully from the high sample-throughput capacity that is possible with LC-MS/MS, automation of sample preparation becomes highly desirable. Unfortunately, liquid-liquid extraction is challenging to automate, and most of the efforts have been directed during the last years towards automation of solid-phase extraction. SPE is mainly performed with extraction cartridges packed with various reversed-phase materials. The plasma sample is applied directly onto a cartridge. Analytes are trapped, while plasma proteins and salts are washed out. Then, the cartridge can be washed with different organic solvent/water mixtures, and finally, the analytes are eluted with organic solvents such as methanol or acetonitrile. Generally, the extract can be injected directly onto the LC column only after dilution with an aqueous solution to reduce the elution strength. When low detection limits are required, an extra evaporation step is mandatory.

The development of 96-well microtiter plates packed with standard solid-phase extraction adsorbents allowed a major advance in high-throughput automated sample preparation [18]. Using a programmable liquid-handling station, a block of 96 samples can be extracted within 10–30 min. This system is not fully automated, and an extra pipetting workstation is needed to dispense the plasma samples into the 96-well plates.

An adaptation of off-line SPE was also made (*Prospekt*), allowing direct fully automated injection of diluted plasma samples onto narrow-bore cartridges [19]. Each sample is injected onto a new cartridge to avoid cross contamination. One limitation of this system is the amount of plasma which can be injected. In addition, such approaches require that the analytes are stable in diluted plasma over several hours.

Pleasance and co-workers developed a custom-built *Zymark* robot system allowing fully unattended sample preparation with SPE or liquid-liquid extraction [20][21]. This complex and impressive system, using off-line batch processing, is well suited for high-throughput laboratories dealing with multiple projects, but requires a dedicated operator for method development and maintenance.

Another efficient and low-cost approach is the use of on-line solid-phase extraction or column switching. Column switching has been a well-established technique applied in LC-UV or LC-fluorescence detection for more than 15 years [22]. The simplest system consists of a trapping column (TC) and an analytical column (AC) connected through a six-port valve. In a first step, the sample is

injected onto the TC using, generally, an aqueous mobile phase where the analytes are trapped onto the TC, while endogenous compounds and salts are washed out. In a second step, the TC is connected to the analytical column (AC), and the analytes are transferred in backflush or frontflush mode to the AC. The system can easily be fully automated. There are several advantages of on-line SPE compared to off-line SPE. First, no evaporation of the extract is required, the whole sample amount is used, and therefore, ultimate sensitivity can be achieved. Second, the same TC can generally be used for several hundred injections, either of plasma or of the supernatant after protein precipitation. Also, the packing material of the TC column is generally of much better quality compared to that used in SPE cartridges, and it can also be easily customized. One of the limitations are cycle times in the range of 7 to 10 min. An elegant and simple solution to improve sample throughput without compromising performance of the assay is to use a dual column switching, as illustrated in *Fig. 3*. In such a system, the second sample can already be injected onto TC2, while TC1 is still connected to the AC. A time gain of 50% can be achieved, as illustrated in *Fig. 4*. For many LC-MS specialists, column switching is wrongly considered as a complicated technique and not being suitable for high throughput. There is still a lot of potential to improve the performance of column switching, and run cycles in the range 2 to 3 min for 2–3 analytes in the pg/ml range have already been achieved [23].

The 96-well plate format is rapidly becoming the standard format in bioanalytics. Recently, *Steinborner* and *Henion* [24] performed liquid-liquid extraction on a 96-well plate format for the determination of methotrexate (MTX) and its hydroxy metabolite (MTX-OH) in human plasma. The lower limit of quantification (LOQ) achieved with ion spray in SRM mode using 0.2 ml plasma was 0.5 ng/ml for MTX and 0.75 ng/ml for MTX-OH with an analytical run time of 1.2 min. Autosampler carry-over limited the achievable LOQ. The fully automated analysis of MTX and MTX-OH using column switching after protein precipitation has been previously achieved with a LOQ of 50 pg/ml for MTX and 200 pg/ml for or MTX-OH using a 0.2 ml plasma aliquot [23].

To benefit fully from automated sample-preparation technologies, it is essential to consider the complete analytical process. Its application is only worthwhile with the 96-well plate format when large sample batches, as encountered in clinical trials, are available, and when sample handling is carried out in conjunction with robots or liquid-handling devices. In addition, data-acquisition and -processing procedures have to be fully automated.

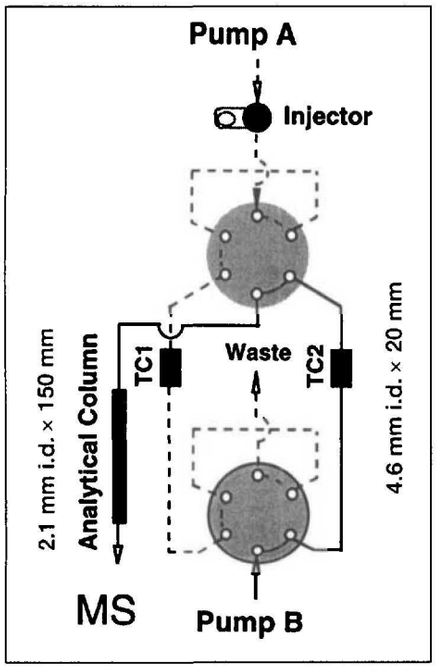

Fig. 3. *High-throughput dual column-switching set-up consisting of two six-port valves, two trappings columns (TC1 and TC2), and one analytical column: TC2 is connected to the analytical column while the next sample can already be injected on TC1*

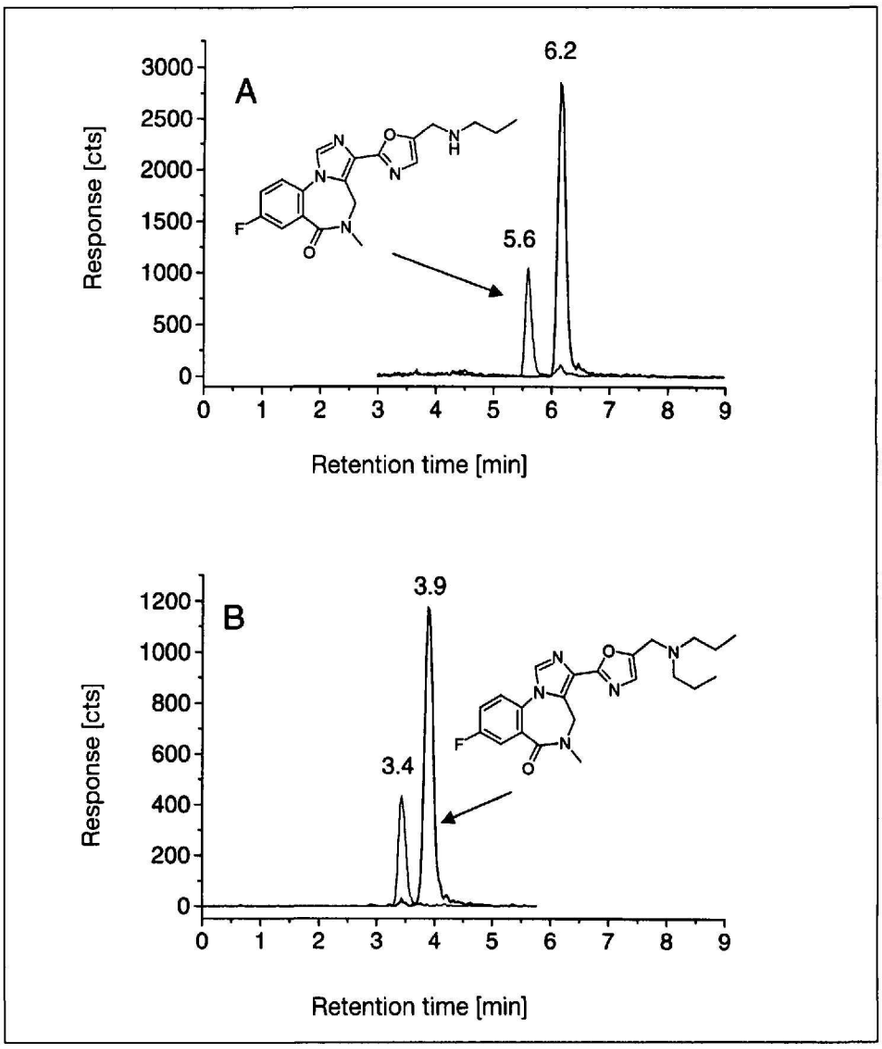

Fig. 4. *SRM Chromatograms for a plasma standard for the metabolite (t_R = 5.5 min) and the parent drug (t_R = 6.2 min) with single (A) and dual column-switching (B) approach*

4. μHPLC-MS/MS

When using a detector in conjunction with a chromatographic separation, it is essential to understand if the detector is mass-flow or concentration-sensitive [25] [26]. In general, a mass spectrometer equipped with electron-ionisation or chemical-ionisation sources behaves like a mass-flow-sensitive detector. In contrast, ion-spray LC-MS behaves as a concentration-sensitive detector where the ion-current response is directly proportional to the analyte concentration in the chromatographic peak, which in turns is dependent on the internal diameter of the analytical column. The sensitivity of a detection system is defined by the signal-to-noise peak-height ratio. Traditionally, LC is performed on standard columns with 4.6 mm i.d. at a flow rate of 1 ml/min. Most LC-MS methods with ion spray are developed for narrow-bore HPLC columns with 2.0 mm i.d. and a flow rate of about 0.2–0.3 ml/min. The use of narrow-bore columns does not only result in lower solvent consumption, but also in a theoretically five-fold increase in sensitivity *vs.* a standard-bore columns when injecting the same amount of analyte. The increase of sensitivity, in the case of extracts from complex matrices, using smaller-bore columns is only realistic when the detection system is very selective. The gain in sensitivity is even greater when using capillary columns with 0.3 mm i.d. at flow rates of 5 μl/min. The injection volume also plays an important role when the overall sensitivity in LC-MS with different column diameters is considered. Injection volumes of more than 50 μl (plasma extracts) are quite common on standard-bore columns, while this volume is limited to less than one microliter when using packed capillary columns. *Zell et al.* [27] have reported an elegant solution to this problem, using column switching to focus a 50 μl liquid-liquid extract of the analytes onto a microbore trapping column (1.0 mm i.d.) followed by backflushing of the analytes onto the packed capillary column (0.3 mm i.d.). With this approach, where column switching is used for injection purposes, they reached a quantification limit of 1 pg/ml using a 1-ml plasma sample. This example demonstrates that chromatography can play an important role in LC-MS for the achievement of ultimate sensitivity. High sensitivity can also be used to minimize sample consumption, which can be very important when performing pharmacokinetic studies with small animals, where only low plasma volumes are available.

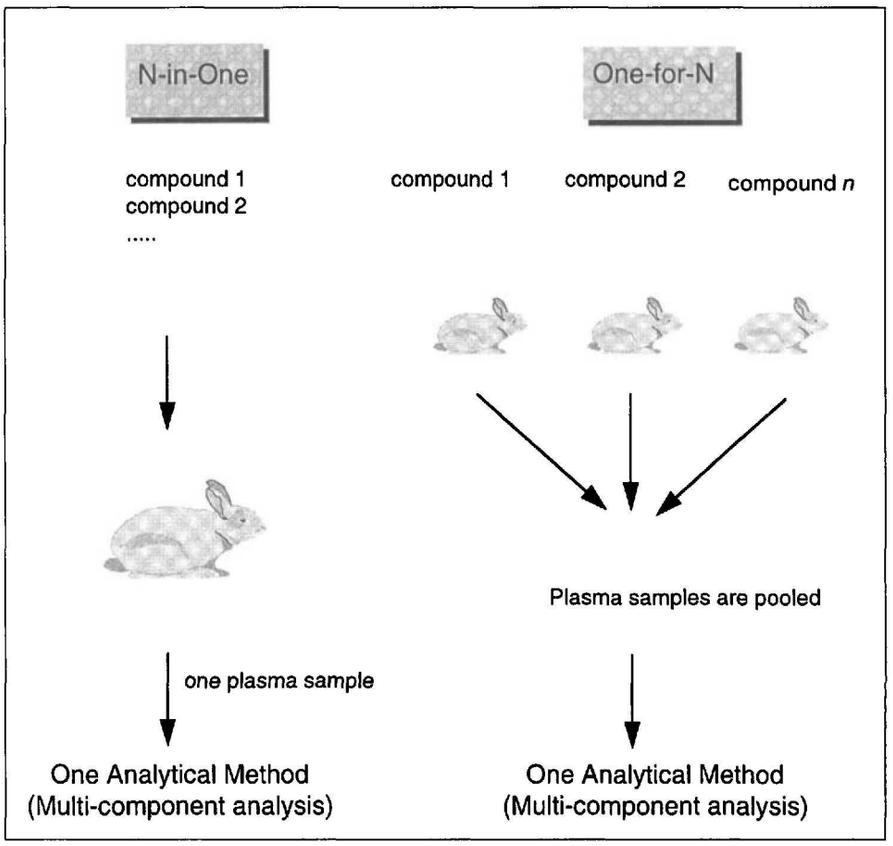

Fig. 5. *Strategies for kinetic* (N-in-One) *and analytical cocktails* (One-for-N). In N-in-one experiments or cassette dosing, several different drugs are administrated simultaneously to the same animal. In oOne-for-N experiments, a single drug is administrated to one animal and the plasma samples are then pooled together to form one plasma sample containing different drugs.

5. Multicomponent Analysis

In the drug-discovery process, LC-MS plays an increasingly important role in the rapid pharmacokinetic screening of a large selection of different compounds. To cope with these new issues, generic bioanalytical approaches are required. The challenge is to develop a single method for several analytes, which have significantly different physico-chemical properties. Due to the unique selectivity of LC-MS, co-eluting analytes can be quantified accurately and precisely, assuming they have different masses or fragments. In a conventional LC-MS method with a single analyte, the MS acquires data only in a 30-s window. There are several ways to use a mass spectrometer more efficiently. The first would be to have several HPLC systems in parallel with multiple sprayers; this, however, is expensive and technically challenging. The second consists of accelerating the HPLC analysis by miniaturization, giving run times in the range of 5 s. This has already been demonstrated, but still requires some further hardware improvements. The third approach is to analyse mixtures of drug candidates. *Olah et al.* [28] described the simultaneous determination of 12 drug candidates in rat and dog plasma over a concentration range 1–1000 ng/ml, using the same assay. This approach was used to support drug discovery by providing pharmacokinetic data, following either after administration of the single analytes and pooling of the plasma samples ('analytical cocktail'), or after administration of multiple compounds ('kinetic cocktail' or 'cassette dosing'), as illustrated in *Fig. 5*. LC-MS/MS analysis was performed in the SRM mode using a triple quadrupole instrument (*PE Sciex, API III*$^+$) equipped with turbo ion spray or an APCI-heated nebuliser. The run-cycle time was less than 10 min. The number of analytes which can be monitored in SRM mode without compromising sensitivity is limited by the mass spectrometer and the data-handling software. The authors have estimated that not more than 25–30 compounds can be measured precisely and accurately in the ng/ml range. Our experience with different projects [29] has shown that this number is strongly dependent on the structure of the selected candidates and is practically limited to 8–10 analytes. In certain cases, it is more efficient to work

with a smaller group of compounds instead of compromising data quality. Currently, the most challenging and time-consuming task is method development and method validation. In any case, this additional work is worthwhile even if the method is only suitable for two drug candidates.

As mentioned earlier, LC-MS/MS analysis can be performed very rapidly, assuming that the complete analytical process from sample delivery in the analytical laboratory to the reporting of the result is optimized. The rate-limiting step is becoming method development. *Beaudry et al.* [30] have reported the application of on-line *Prospekt* solid-phase extraction combined with LC-MS/MS in the APCI mode to support *in vivo* PK screening cassette-dosing experiments in a fully automated mode. The TQ was an *API 3000* with a newly designed collision cell allowing very fast SRM experiments in the range of 10 ms per transition. They applied their approach to a *n-in-one* experiment for 64 generic pharmaceutical compounds. A LOQ of 0.5 ng/ml with a dynamic range of three orders of magnitude has been achieved with correlation coefficients in the range of 0.985–0.998. Unfortunately, no precision or accuracy data were reported.

Considerable progress has been made with LC packing material over the last few years. As illustrated in *Fig. 6*, the SRM/LC-MS analysis of 25 different acidic, neutral, and basic compounds, with calculated octanol/water partition coefficient (ClogP) in the range –0.2 to 8 demonstrates good chromatography-peak shape for almost all compounds using a fast gradient starting from a high aqueous content to 100% acetonitrile within 2 min [31]. A dwell time of 10 or 40 ms was used for each transition. A 20-µl injection of standard solutions covering the range from 0.1 to 100 ng on column gave good accuracy and precision for almost 24 compounds, with a dynamic range of three orders of magnitude. For each compound, a single method has already been successfully developed with a LOQ below 1 ng/ml. A generic sample-preparation method based solely on protein precipitation and direct injection of spiked human plasma samples in the concentration range of 1–1000 ng/ml, showed that a LOQ of 1 ng/ml could be achieved only for one analyte with acceptable precision and accuracy. For all the other compounds, the LOQs were found to be in the 20–100 ng/ml range.

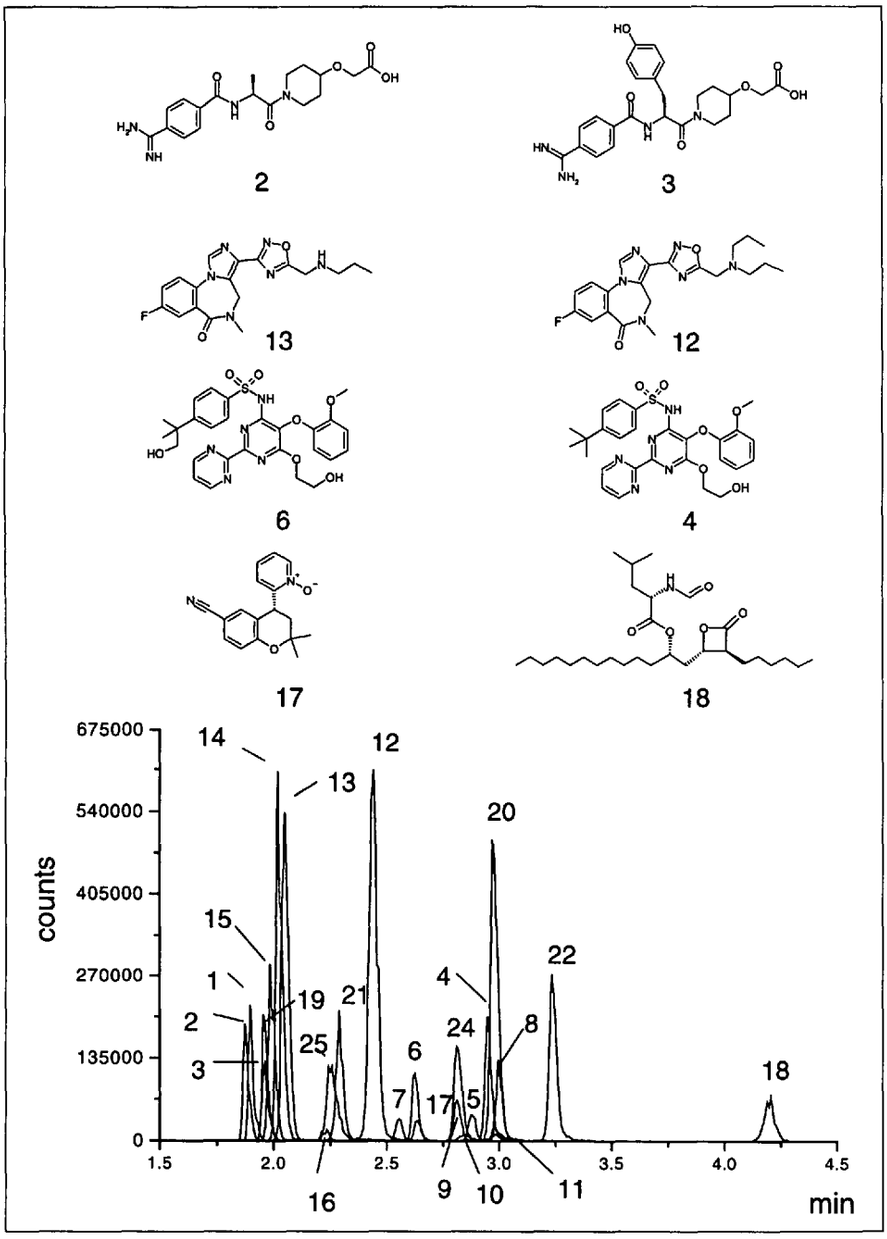

Fig. 6. *SRM/LC-MS Analysis onto a narrow-bore analytical column* (cocktail of 25 different compounds; 2.5 ng on-column of each analyte)

6. CZE-MS

Capillary zone electrophoresis (CZE) is a high-resolution separation technique, where the analytes migrate through a capillary filled with a background electrolyte with constant but different mobility, and the separation is achieved by differences in the mobility of the analytes. One million theoretical plates can easily be achieved. Combination of CZE with mass-spectrometric detection was also highly desirable [13][14]. Electrospray works best with ions in solution, as is also the case for CZE. Unfortunately, the other parameters for both techniques are less compatible, in particular, the type and concentration of the background buffer. Various interfaces have been described, including the liquid-junction interface, the sheath-liquid interface and the sheathless interface. Recently, *Hewlett-Packard* proposed a commercial product for CZE-MS with a single quadrupole and ion-trap (IT) MS. Currently, most activities are carried out with home-made interfaces, and the coupling of CZE with API remains a challenging task compared to µLC-MS. It is particularly problematic for quantitative analysis in biological fluids.

With LC, it is sometimes difficult to retain highly charged analytes. Due to the different separation mechanism for CZE, the elution order within a mixture is differ-

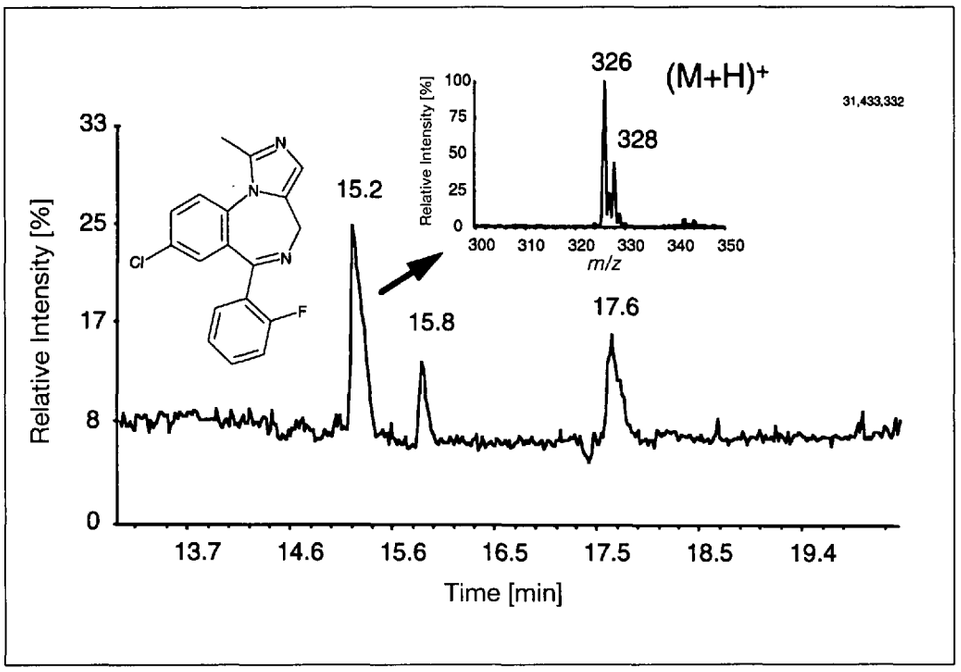

Fig. 7. *CZE-MS Analysis of midazolam and several metabolites.* Electrophoretic separation was achieved on an 0.1 mm i.d. × 1 m length fused-silica capillary at a potential of 13 kV using the sheath-flow interface. The buffer consisted of 20 mM ammonium acetate buffer at pH 4.6. The sheath liquid infused at 20 ml/min consisted of a mixture of methanol/5 mM ammonium acetate/acetic acid (80/20/1, *v/v/v*).

ent from HPLC. This feature is interesting for qualitative analysis, particularly for polar phase-II metabolites, as encoutered for midazolam or tolcapone, where a glucuronide conjugate migrated more slowly than the parent drug.

Lausecker et al. [32] have investigated the benefits and drawbacks of CZE-MS *vs.* μHPLC-MS for the quantification of midazolam in human plasma at the low ng/ml range on a triple quadrupole instrument using a home-made sheath-liquid interface. CZE-MS showed superior sensitivity, however, the sample-preparation step remained a bottleneck. They found that SPE was not suitable for human plasma, and a more selective liquid-liquid sample-preparation procedure was necessary.

Bach and *Henion* [33] developed a CZE-MS method for the quantification of methylphenidate in a 4-ml urine sample, using a LCQ ion-trap mass spectrometer. They applied liquid-liquid extraction and achieved a LOQ of 1.5 ng/ml. The LCQ is capable of acquiring full-scan data and MS^2 or MS^n within a single duty cycle, which allows, besides quantification of the analyte, screening for other compounds. This powerful feature is unique to IT instruments.

The application of CZE-MS to the determination of paracetamol and its metabolites in urine and serum has been described by *Blaschke* and co-workers [34]. In order to prevent peak broadening, a water zone was injected before and after the samples. They performed various simple sample-preparation steps, including dilution, protein precipitation, and ultrafiltration. Quantification limits for paracetamol of 7.5 μg/ml for urine and of 1.25 μg/ml for serum were achieved. This example illustrates the current limitations of CZE in bioanalytics. Unfortunately, μg/ml sensitivity is not sufficient to support development of new drug candidates. Sample-concentration techniques have been described, but their use is still limited [35–37].

CZE has been carried out primarily with aqueous electrolyte buffers. For non-polar compounds, micellar electrokinetic chromatography (MEKC) is the method of choice, but the surfactants used with this technique suppress the electrospray response dramatically. An exciting emerging alternative to MEKC is the application of a non-aqueous electrolyte buffer (NAC-ZE) to the separations of more lipophilic, small pharmaceutical molecules, which is fully compatible with electrospray ionization [38].

Although the coupling of capillary electrochromatography (CEC) with electrospray has already been described in 1993 [39], this technique is still under development, but certainly offers a great potential.

CZE and CEC generate peaks with peak widths of a few seconds. In the full scan mode, quadrupole instruments have a typical 1-s scan time for a mass range of 1000 amu. Therefore, not enough points can be collected with these types of instruments. This proble is illustrated in *Fig. 7* for the full-scan CZE-MS analysis of a 1-ng midazolam peak. The full scan spectrum can only be recorded in a very small mass window without compromising sensitivity. Quadrupole ion trap and time-of-flight (TOF) mass analysers, which can record full-scan spectra over a large mass range within 100–200 ms, are more suitable mass analysers, for CZE-MS or CEC-MS. Another limitation of CZE-MS is still the physical size of the commercially available instruments, which are simply too big. It is technically difficult to achieve separation with capillaries shorter than 30–40 cm. One of the solutions is miniaturisation on a chip base, where the electrosprayer is integrated in the chip.

7. Single-Quadrupole *vs.* Triple-Quadrupole Systems for Quantitative Analysis

In pharmaceutical drug research and development, most of the quantitative assays reported are based on complex and expensive triple quadrupole mass spectrometers. In many laboratories, a shift from traditional LC-UV to LC-MS/MS methods has occurred. With a triple quadrupole instrument, ultimate sensitivity and selectivity can be achieved with very short analysis times, assuming that no significant matrix suppression occurs during the ionisation process. More recently, low-cost single-quadrupole instruments (SQ) became available using the same interface design as the larger TQ instruments. Similar sensitivity should be expected with single-quadrupole devices in the selected-ion monitoring mode (SIM) as for triple-quadrupole instruments in selected-reaction monitoring mode (SRM). The actual

lower limit of quantification (LOQ) with biological extracts using SIM/LC-MS is strongly dependent on the chromatographic and sample-preparation selectivity. In the development phase of a new drug candidate, a pg/ml sensitivity may not be required at all stages. SIM/LC-MS is then a cheaper alternative to SRM/LC-MS, and a more efficient and reliable alternative to LC-UV, in particular for large clinical studies where the patients often have co-medication. This has recently been demonstrated for sibrafiban, where, at the start, a sensitive assay was required to support PK studies in animals [40] with a LOQ of 0.2 ng/ml, and, at a later stage, a single LC-MS was established to support the analysis of twenty-thousands of samples with a LOQ of 1 ng/ml [41]. With modern TQ instruments, LOQs down to 0.1 ng/ml are very common. Generally, LOQs down to 1 ng/ml for many compounds can be achieved reasonably without compromising analysis time with SIM/LC-MS, which is sufficient for many studies.

One of the unique features of the API source is its capability of performing fragmentation of the protonated or deprotonated molecular ions by collision-induced dissociation (CID) before the first mass-analysing quadrupole (Up-front CID). The mass spectra, which are obtained by collision of the ions with a neutral gas, mainly nitrogen, are very similar to those obtained by low collision energy in a collision cell of a triple quadrupole instrument. Up-front CID can be exploited either to obtain structural information, or to generate confirmation ions in quantitative analysis with single MS, which increases reliability of the results, or to monitor possible interactions of endogenous or co-administered drugs when samples from completely different species and study designs are analysed. The comparison between SRM/LC-MS and SIM/LC-MS for the analysis of compound IV in rat plasma is illustrated in *Fig. 8A–D*. Chromatographic separation was performed isocratically at a flow rate of 0.2 ml/min on an *Inertsil ODS-2* 2.1 mm i.d. × 100 mm length column. The mobile phase was MeOH/H_2O/1% HCOOH (85/15/1, *v/v/v*). The analyte and the internal standard were isolated from 100 µl rat plasma by liquid-liquid extraction with a mixture of ethylacetate/hexane (50/50, *v/v*). Mass-spectrometric analysis was performed on an *API 3000* (*PE Sciex*), either in the SIM or in the SRM mode. The product-ion spectrum of the compound shows a major fragment at *m/z* 373. In the SIM mode, this fragment was monitored by setting the orifice voltage at –105 V, while the protonated molecule was monitored at an orifice potential of –45 V. *Fig. 8A* shows the extracted ion-current profile for the analytes and the IS for a blank rat-plasma sample in the SRM mode (left) and in the SIM mode (right). The superior selectivity of SRM/LC-MS *vs.* SIM/LC-MS is obvious. *Fig. 8B* shows the analysis of a rat-plasma sample from a pharmacokinetic study. Very similar concentration values are obtained in SRM/LC-MS and SIM/

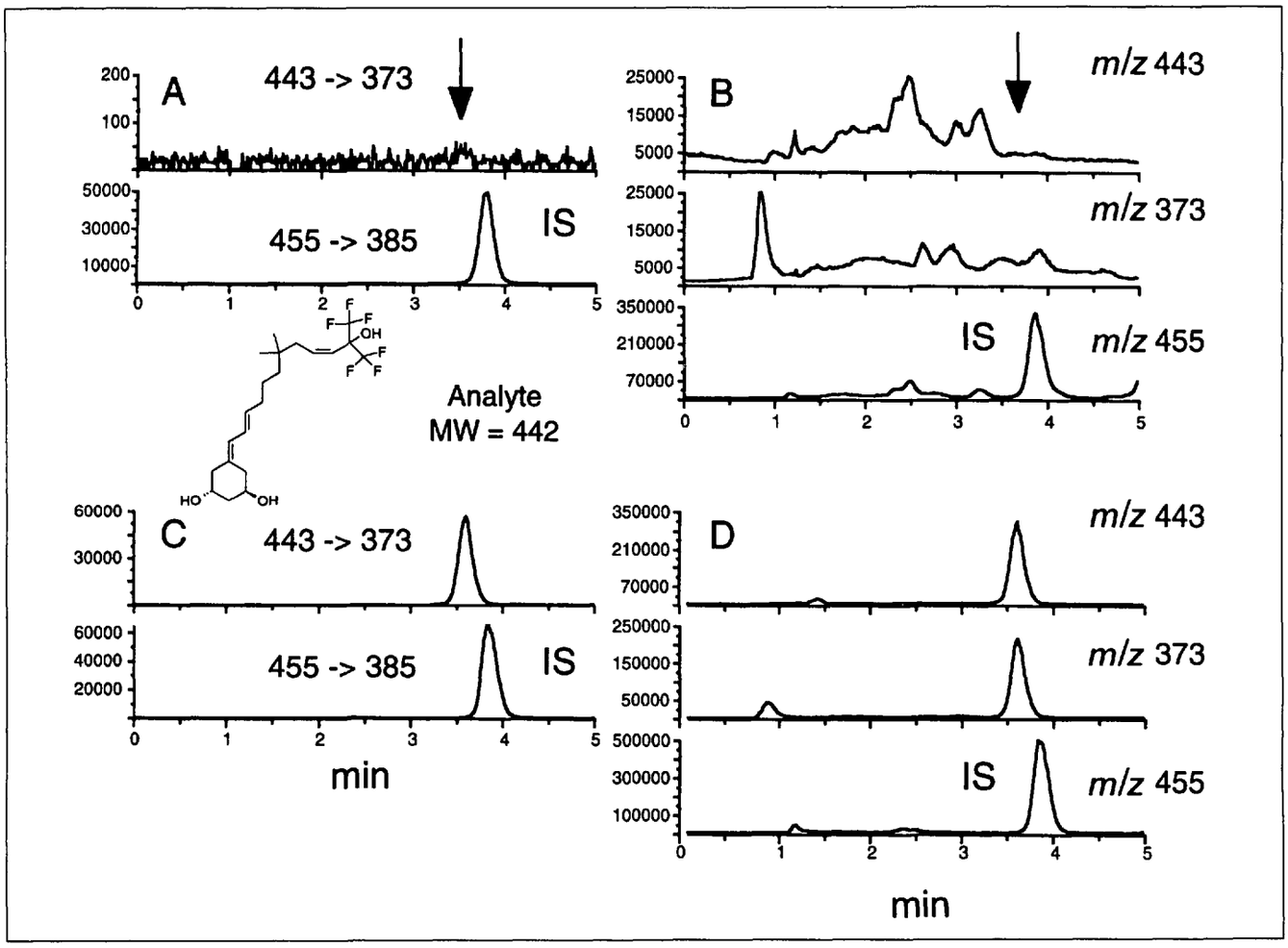

Fig. 8. *Analysis of the same plasma sample performed either in the SRM/LC-MS (left) and SIM/LC-MS (right) modes.* Blank rat plasma with IS *A*) SRM/LC-MS *B*) SIM/LC-MS. Rat-plasma sample after oral administration of the analyte IV *C*) SRM/LC-MS *D*) SIM/LC-MS. The trace at *m/z* 373 in the SIM mode corresponds to the confirmation ion.

LC-MS mode. In addition, a peak corresponding to the confirmation ion is observed at *m/z* 373 in SIM/LC-MS. The ratio between *m/z* 443 and 373 is constant and independent from the concentration of the analyte. It depends only on the setting of the orifice voltager and the instrument type. This ratio can therefore be used to monitor possible interference with others endogenous compounds. The ratio was found to be 1.5 between the peak area of the ion at *m/z* 443 and the ion at *m/z* 373. The average peak-area ratio, from 12 rat-plasma samples in the concentration range 6 to 120 ng/ml from a pharmacokinetic study, was found to be 1.48 with a relative standard deviation of 1.73 %. Peak-ratio changes over 20–30% are considered to be significant and indicates, the presence of an interfering compound. This approach is not possible with analytes which are generating many fragments, but it should be always considered because of its simplicity and the extra quality gained. Quadrupole ion traps are an interesting alternative to TQ instruments. They have also MS^n capability, which is particularly useful for qualitative analysis. Quantitative analysis has been demonstrated on IT, however, quadrupole instruments remain the work horses for quantitative analysis

8. Conclusion

LC-MS is a well-established technique for bioanalytical applications in the pharmaceutical industry allowing to perform the analysis of drug candidates in biological fluids in a much faster, more sensitive and accurate way than in the past. Automation of sample preparation as well as data processing will become increasingly important. Limitations such as matrix suppression in the ionisation process of API sources have been recognized and can be overcome when an adequate sample-preparation strategy for a given problem is selected. The next step in this field will certainly be miniaturisation. Techniques such as CZE and CEC may find wider application in the future when LC-MS goes on a chip.

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