REVIEW

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Quantitative analysis of pharmacokinetic study samples by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

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Dedicated to Prof. Dr. S. Ebel, Würzburg, on the occasion of his 65th birthday

The basis of all pharmacokinetic evaluations are powerful assays to quantify drugs and/or metabolites in biological matrices using modern sensitive instrumental analytical techniques, such as capillary gas chromatography and high-performance liquid chromatography (HPLC). Being both specific and universal, mass spectrometry (MS) is an ideal chromatographic detector. Due to recent exciting achievements in the interfacing of liquid chromatography (LC) and MS, LC-MS, like the successfully preceding hyphenated technique gas chromatography-mass spectrometry (GC-MS), has now become a valuable technique in the analyst's toolbox. The key features of LC-MS are explained and four examples demonstrating its potential for highly specific and sensitive routine drug assays with the option of high sample throughput in pharmacokinetic investigations are presented.

1. Introduction

The efficacy and safety of many drugs is related to the concentrations of drugs and/or metabolites in body fluids and tissues. Consequently, essentially all clinical pharmacological investigations and most clinical trials require the analysis of drugs and/or metabolites in biological samples. Accurate data on drug and metabolite concentrations in body fluids and tissues are the basis of pharmacokinetic evaluations and pharmacokinetic/pharmacodynamic correlations which then facilitate a proper choice of dose, optimum dose frequency and drug formulation. Possible alterations in pharmacokinetics due to age, gender, ethnic factors, concomitant drugs or diseases are further important issues to be clarified during clinical drug development − all dependent on high-quality bioanalytics.

2. Bioanalytical requirements for pharmacokinetic/bioavailability investigations

Owing to the enhanced potency of modern drugs and subsequently low doses, assays with high detection sensitivity are being required more and more frequently, with limits of quantification (LOQ) in the nanogram per litre range. Such trace analysis in complex sample matrices excessively challenges assay specificity.

High concentrations at peak as well as the elimination characteristics, with drug concentrations approaching LOQ, are of interest for a new drug compound, leading to an assay working range often comprising several orders of magnitude. As the quality of the pharmacokinetic data is directly dependent on the accuracy of analytical data, their quality, i.e. accurate and precise quantitative results, has to be rigorously verified by intensive method validation and quality control procedures. Both EU and FDA have published respective guideline [1, 2], defining the method specifications, validation procedures and quality control data during routine analysis to be well documented and presented in a new drug application package.

As the once established assays are generally used for thousands of clinical study samples in all phases of clinical drug development, which may last many years, aspects of sample throughput (time & cost aspects), ruggedness and ease of method transfer, are additional issues to be considered during method development.

3. Coupling of HPLC with MS/MS

The analysis of complex mixtures by means of chromatographic separation techniques has been state-of-the-art for years. In the biomedical-pharmaceutical sciences high performance liquid chromatography (HPLC) is of particular importance as drugs are usually non-volatile, thermolabile, polar, etc., i.e. generally not amenable to gas chromatography (GC). The most popular detecting device in HPLC is the UV-detector recording the absorption of ultraviolet light by the eluate. However, as assays with low LOQ are being required more frequently, UV-detection, also suffering from low specificity (dependent on the chemical structure of the analyte), is increasingly approaching its limits. Fluorescence and amperometric detection are extremely useful for bioanalytical applications, their use, however, is limited to drugs with native fluorescence or suitable functional groups to be oxidized, unless preceding derivatization compensates for this lack of universality.

Mass spectrometry (MS) detects ionised organic molecules in the gaseous phase (at high vacuum of approximately 10^{-5} Torr) as a function of their mass-to-charge ratio, which in turn is directly dependent on the molecular weight. MS is indispensable for the identification and structure elucidation of organic compounds. It is also an ideal detector for chromatography, being not only specific and universal but at the same time usually showing a high detection sensitivity. This ideal coupling of two powerful analytical techniques is state-of-the-art in gas chromatography mass spectrometry (GC-MS). Despite the technical difficulties of the antagonism between liquid effluent of the HPLC column and high vacuum of MS, interfaces overcoming this hurdle are now commercially available and have allowed LC-MS advance to maturity [3−5]. The preferred tools today are [6, 7]: Atmospheric Pressure Chemical Ionization (APCI) and Electrospray (ES).

With APCI (Fig. 1) volatilization of the HPLC effluent is accomplished by the combination of heat and gas flow

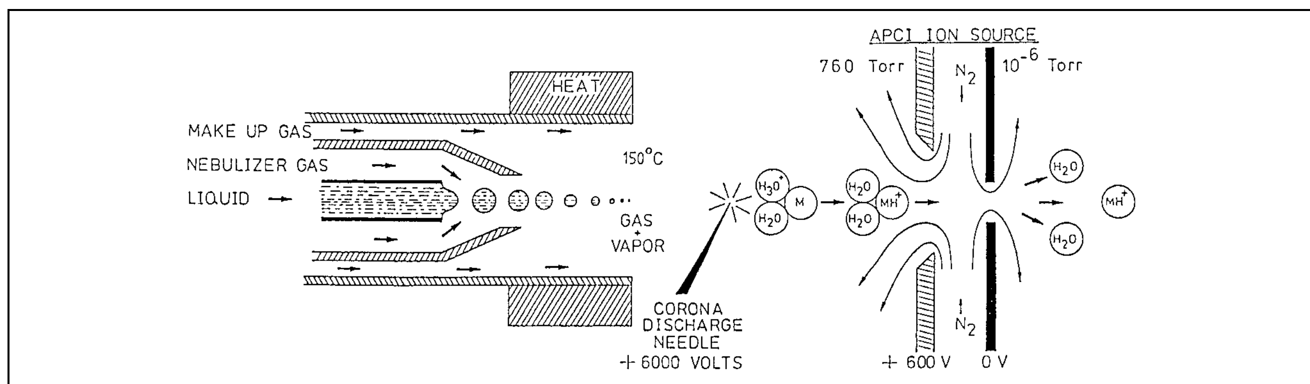


Fig. 1: Schematic diagram of atmospheric pressure chemical ionization (APCI) (reprinted with permission from Covey et al.: Anal. Chem. **58**, 1451A (1986). Copyright 1986 American Chemical Society)

within the atmospheric pressure ion source. Ionization of solvent molecules is initiated by a corona discharge at the discharge needle; the solvent ions then produce analyte ions by chemical ionization at atmospheric pressure of the analyte. The most attractive feature of this interface is its capability to handle flows ranging from 0.5–2 ml/min, compatible with conventionally-sized HPLC columns (4.6 mm inner diameter).

The most interesting recent development in the interface field, however, has been electrospray where liquid in a narrow capillary (50–100 μm inner diameter) produces a fine mist of droplets at the exit when a high voltage of several kilovolts is applied (Fig. 2). By adding nebulizing gas and/or heat finely dispersed and stable sprays at flow rates of 50–1000 μl/min compatible with HPLC are possible.

Applying a high voltage to the capillary not only produces an aerosol of fine droplets, but also charges the droplets. Therefore, ions can evolve from these charged droplets when they are shrinking in size on their way through the atmospheric pressure ionization chamber. This process [8], called "field-assisted ion evaporation", is a very mild form of ionization which is ideal for labile, polar and ionic species (e.g. biomolecules as peptides, proteins and DNA, drugs, etc.).

Besides the advantage of being an universal detector with high detection sensitivity, LC-MS can be very specific. This can lead to a simplification of working processes and savings in sample preparation and chromatography, coupled with a high sample throughput in routine analysis.

This holds especially true, when a tandem mass spectrometer (MS/MS) can be used [9]. As both APCI and ES provide simple MS spectra, often only showing the (pseudo)molecular ion (M^+, M^-, $(M + X)^+$, where $X = H$, NH_4, Na, etc.) as base peak, this signal (i.e. information) concentrated in one ion species may be subjected to collision-induced dissociation with a collision gas (see Fig. 3 and example spectra in Fig. 4). The subsequent fragmentation of the parent ion into daughter ion(s) is again indicative of the chemical structure of the analyte, increasing the specificity of the finally observed signal of this 'selected reaction monitoring' (SRM). Thus, LC-MS/MS ideally complements the mild ionization conditions afforded by both API-MS techniques with the – compared to capillary GC – limited separation efficiency of HPLC.

Finally noteworthy to mention, MS detection also allows the use of stable isotope-labelled compounds (deuterium, ^{13}C, ^{15}N) as internal standard as well as employing stable isotope-labelled drugs directly for specific bioavailability determinations [10], drug formulation comparisons [11] and metabolism investigations [12].

Since the pioneering work of Covey et al. using LC-MS/MS for quantifying drug plasma concentrations in a pharmacokinetic study [13], LC-MS/MS nowadays plays a vital role in bioanalytics for pharmacokinetic investigations, enabling assays for drugs in fluids to be developed in days rather than weeks and permitting batch processing with rapid sample throughput and sensitivities previously attainable only after tedious efforts [14]. The following examples covering various of the favourable features of LC-MS/MS in assay development and routine bioanalysis may help to explain this attractiveness and oustanding success of LC-MS/MS in the field of pharmacokinetic investigations.

3.1. Example 1: high detection sensitivity

Previous investigations of biological disposition and percutaneous absorption of the imidazole antimycotic bifonazole in man have used radiometric methods or a specific high-performance thin layer chromatographic (HPTLC) assay with postchromatographic derivatisation. The LOQ

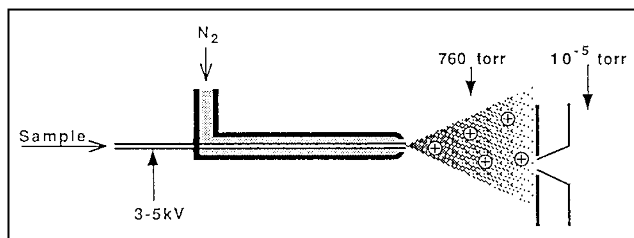


Fig. 2: Schematic diagram of pneumatically-assisted electrospray (reprinted with permission from Huang et al. Anal. Chem. **62**, 713A (1990). Copyright 1990 American Chemical Society)

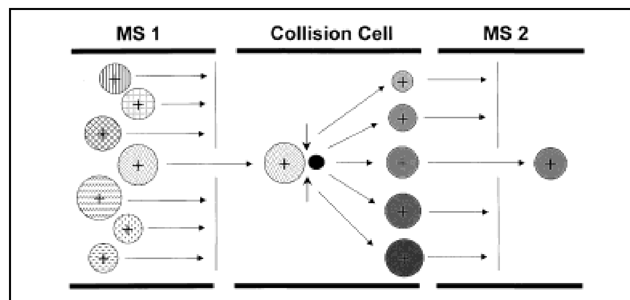


Fig. 3: Schematic layout of tandem mass spectrometer (MS/MS) operated in the 'selected reaction monitoring (SRM)' mode: analyte ions are filtered in the first mass spectrometer (MS 1), subjected to collisions with gas atoms leading to characteristic fragments, which are then selected in MS 2

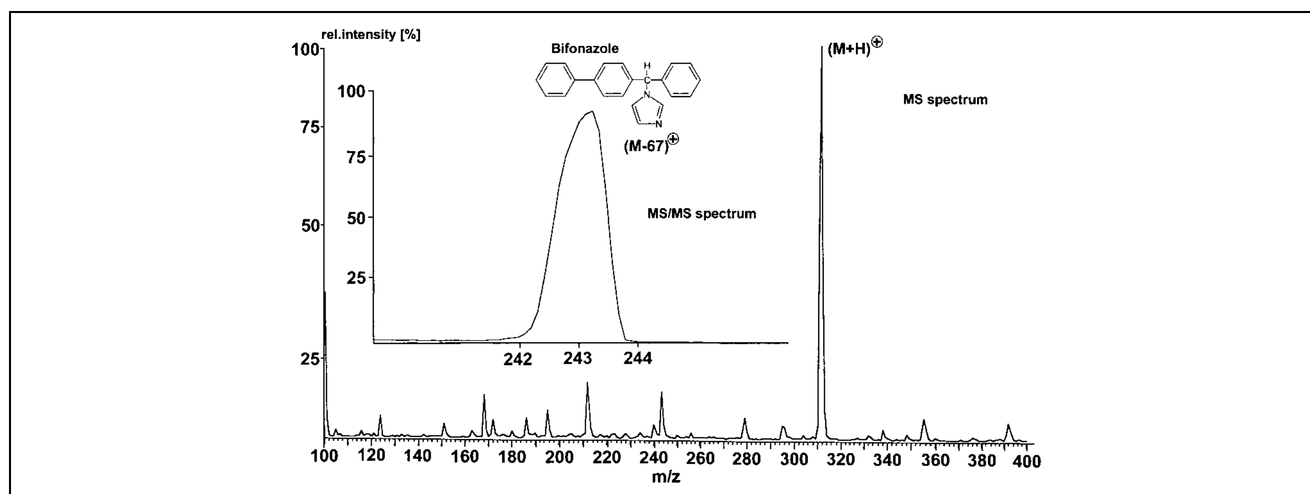


Fig. 4: Bifonazole electrospray MS spectrum (with base peak at m/z 311 $[M + H]^+$) and MS/MS spectrum (with base peak at m/z 243 $[M\text{-}67]^+$ due to loss of the imidazole ring by collision-induced dissociation)

achieved was at 1.0 µg/l [15]. Recent assays published for other azole antimycotics such as clotrimazole and miconazole, for instance, were all based on HPLC or GC methods resulting in comparable LOQs at low µg/l-levels.

For the determination of bifonazole systemic exposure following the topic administration of a newly developed scalp gel to be used against seborrhoic dermatitis, a highly specific and sensitive LC-MS/MS assay has been developed. Bifonazole was extracted from 1.0 ml human plasma with diethylether/n-heptane (1 : 1 v/v) and determined by rapid reversed-phase chromatography coupled with APCI MS-MS detection (Fig. 4). Assay development and standard validation package could be completed within four weeks, including a complete stability programme (stock solution, short-term and freeze-thaw stability in plasma, autosampler and long-term stability in plasma up to several weeks). The straightforward liquid-liquid sample clean-up, easy to automate [16], and a short LC run time of less than 2 min allowed a high sample throughput. The LOQ was 5 ng/l with an accuracy of $\leq \pm 10\%$ and a precision of $\leq 10\%$ ($<20\%$ close to the LOQ) in the working range of 5 ng/l to 10 µg/l [17].

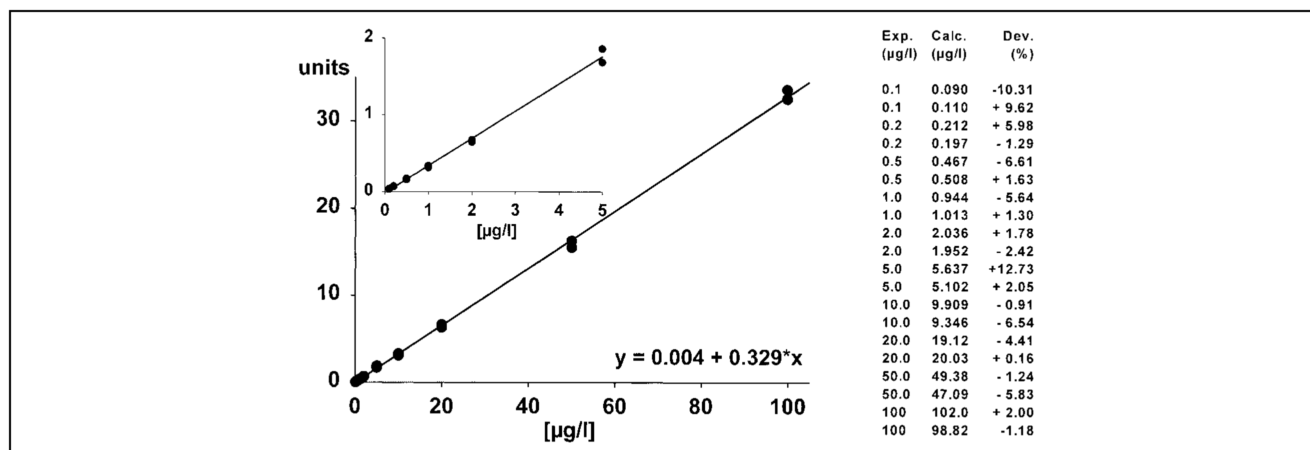
Geometric mean plasma concentrations 1 to 8 h post-dose, following daily morning administrations of 10 g scalp gel 1% (two administrations of 5 g each for 5 min) for five days, were in the range of 0.024 to 0.062 µg/l on day 1 and in the range of 0.15 to 0.18 µg/l on day 5, well below the LOQ of the former procedures [17].

3.2. *Example 2: fast method development*

A rapid assay development of a back-up compound for the calcium promoter BAY y 5959 [18], based on the existing LC-MS/MS procedure for BAY y 5959 (i.e. liquid-liquid extraction; APCI/SRM) was required to investigate the bioavailability of the new drug candidate. The following actions were carried out:

- MS optimization for suitable parent/daughter ion combination for SRM and for highest detection sensitivity (variation of voltages along the ion path, collision energy): 1 day (for one technician at one instrument)
- check for optimum liquid-liquid extraction efficiency: 1 day
- assessment of calibration and quality control procedures: 1 day
- pre-study validation: 3 days
- stability investigations (work-bench, autosampler, storage, etc.) in parallel for 2 weeks.

After exactly two weeks' time the complete assay was ready for use. Typical calibration and the pre-study validation results for the drug and its major metabolite are presented in Fig. 5 and Table 1. Three days after the study, comprising approximately 300 plasma samples, the pharmacokinetic evaluation could be presented for decision-making.



Exp. (µg/l)	Calc. (µg/l)	Dev. (%)
0.1	0.090	-10.31
0.1	0.110	+ 9.62
0.2	0.212	+ 5.98
0.2	0.197	- 1.29
0.5	0.467	- 6.61
0.5	0.508	+ 1.63
1.0	0.944	- 5.64
1.0	1.013	+ 1.30
2.0	2.036	+ 1.78
2.0	1.952	- 2.42
5.0	5.637	+12.73
5.0	5.102	+ 2.05
10.0	9.909	- 0.91
10.0	9.346	- 6.54
20.0	19.12	- 4.41
20.0	20.03	+ 0.16
50.0	49.38	- 1.24
50.0	47.09	- 5.83
100	102.0	+ 2.00
100	98.82	-1.18

Fig. 5: LC-MS/MS calibration data in the working range from 0.1 to 100 µg/l for drug candidate of example 2

Table 1: Pre-study validation results from example 2

	Drug				Metabolite			
Nominal concentration (μg/l)	blank	2.0	20.0	80.0	blank	2.0	20.0	80.0
Measured concentration (μg/l)*	<0.1	1.86	18.8	72.1	<0.1	1.86	18.5	78.4
Accuracy, presented as deviation from the nominal value (%)	–	−6.9	−5.9	−9.9	–	−7.2	−7.4	−2.0
Precision, presented as coefficient of variation (%)	–	4.5	5.2	5.6	–	6.7	7.0	8.3

* mean of 18 determinations: samples were processed by three different technicians (N = 6 each) on two different days

3.3. Example 3: high sample throughput

This example illustrates not only the potential of LC-MS/MS for high sample throughput in sensitive routine analysis, but also for assay selectivity enabling the simultaneous quantitation of drugs and their metabolites owing to the use of MS detection. The assay was developed to determine the dihydropyridine calcium antagonist nimodipine and four of its metabolites which were of toxicological interest in 0.5 ml human plasma. Further method requirements were:

- working range for all five analytes from 0.1 μg/l (LOQ) to 100 μg/l
- accuracy $\leq \pm 10\%$ over the complete working range and precision $\leq 10\%$ (20% allowed close to the LOQ).

A simple liquid-liquid extraction procedure for sample clean-up was possible. The chromatographic separation (with a 40 × 2 mm i.d. C_{18} column as stationary phase and a mixture of acetonitrile/ammonium acetate 10 mM adjusted to pH 3 with formic acid [70:30 v/v] as mobile phase) was deliberately reduced, only a slight separation of the breakthrough peak comprised of salts, proteins and other endogenous compounds with good water-solubility often suppressing sensitivity in electrospray ionization, was accomplished. The effluent entered the tandem mass spectrometer via the pneumatically-assisted electrospray interface [19]. All analyte components were quantified at their specific mass-to-charge ratios (m/z).
The total run time of each injection was less than 2 min, allowing each series, including 22 calibration samples + 8 quality control samples + 60 study samples, to be completed within approximately 3 h (Fig. 6). As the clean-up was easy and suitable for further automation, a high sample throughput in routine use was possible for this selective and sensitive assay.

3.4. Example 4: assay specificity

With its asymmetrical substitution at the dihydropyridine ring the calcium antagonist nimodipine is a racemate. A stereospecific LC-MS/MS assay based on liquid-liquid extraction, separation of racemic nimodipine into its enantiomers via chiral stationary-phase HPLC, collection of the two isomer fractions followed by off-line GC-MS quantification, has been reported [20, 21].
To increase sample throughput, especially by simplifying this tedious and time-consuming procedure and reducing manual operations, a direct HPLC assay using MS/MS detection in the SRM-mode via pneumatically-assisted electrospray has been developed. Routine determination of nimodipine enantiomers in human plasma in the working range of 0.5–75 μg/l plasma for each isomer with accuracy $\leq \pm 10\%$ and precision $\leq 10\%$ (20% close to the LOQ) was possible (Table 2). Compared to the LC-GC/MS assay, the time required for tedious routine analysis of approximately 150 clinical study samples could be reduced by a factor of 4, without comprising sensitivity or specificity of the assay [22].

4. Conclusion

Due to the complementary nature of LC and MS, using powerful interfaces as APCI and ES providing mild but efficient ionization conditions, and MS/MS-detection in the SRM-mode, LC-MS/MS is an ideal "hyphenated" technique for quantitative analysis, offering:

- unique specificity,
- superior LOQ,
- fast method development, and
- high speed/high sample throughput analysis.

Especially, the generally superior LOQs allow straightforward, fast assay development – not restrained by continuously keeping an eye on detection specificity and sensitivity. Actually, the opportunity for a "generalized" method development is given, based on a set of sample pretreatment/clean-up procedures (such as dilution and direct in-

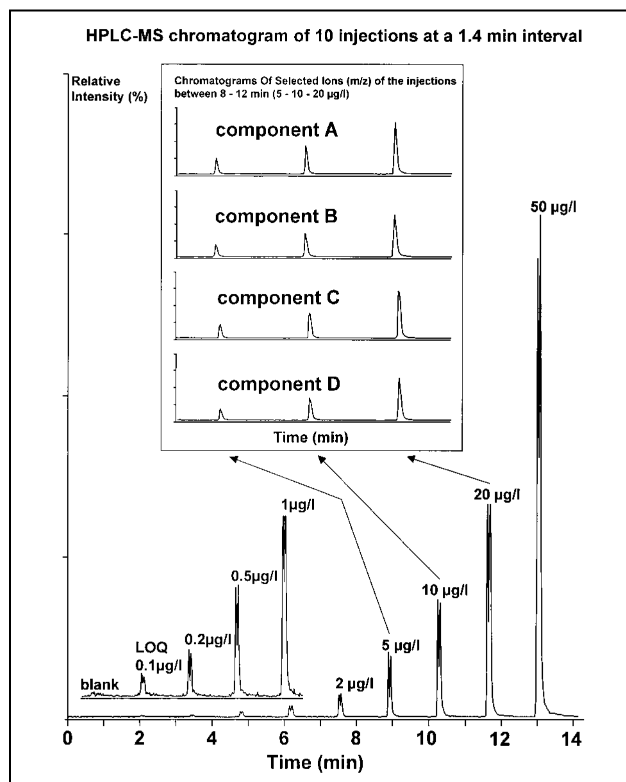


Fig. 6: LC-MS chromatograms for drug metabolites of example 3: ten injections within 14 min

Table 2: Inter-assay accuracy and precision from example 4

Enantiomer	S-(−)	R-(+)	S-(−)	R-(+)	S-(−)	R-(+)
Nominal concentration (μg/l)	50.0	50.0	25.0	25.0	2.50	2.50
N	15	15	16	16	16	16
Mean (μg/l)	47.6	49.0	24.3	24.0	2.46	2.49
Accuracy (%)	−4.9	−1.9	−2.8	−4.2	−1.4	−0.3
Precision (%)	9.0	10.7	7.9	10.2	13.7	11.7

Enantiomer	S-(−)	R-(+)	S-(−)	R-(+)	S-(−)	R-(+)
Nominal concentration (μg/l)	0	60.0	0	18.0	0	5.40
N		15		16		16
Mean (μg/l)	<0.25	55.8	<0.25	17.5	<0.25	5.2
Accuracy (%)		−7.0		−2.9		−2.9
Precision (%)		8.4		5.7		10.8

Enantiomer	S-(−)	R-(+)	S-(−)	R-(+)	S-(−)	R-(+)
Nominal concentration (μg/l)	60.0	0	18.0	0	5.4	0
N	16		16		16	
Mean (μg/l)	59.7	<0.25	17.7	<0.25	5.5	<0.25
Accuracy (%)	−0.6		−1.9		−2.3	
Precision (%)	9.9		8.2		10.1	

jection e.g. for urine samples or injection after ultrafiltration, protein precipitation, liquid-liquid extraction, solid-phase extraction) + high speed reversed-phase chromatography + tandem mass spectrometric detection via the SRM mode.

Owing to its high specificity the use of LC-MS/MS in quantitative bioanalysis allows reduced instrument run times to be typically less than 5 min. Sample preparation, however, has now become one of the rate determining steps to speed up analysis time, next to unified data flow strategies from sample entry up to result exit. To fully exploit the potential of LC-MS/MS for fast routine analysis with high sample throughput a high degree of automation in the (often simplified) sample clean-up/pretreatment is necessary to supply enough samples for injection. Otherwise, the fast instrument run times are only misleading with respect to savings in total analysis time. Direct injection following protein precipitation (plasma samples) or dilution (urine samples), coupled-column-switching and automated liquid-liquid or solid-phase extraction, especially in combination with 96-well microtiterplates, are currently the most popular concepts to address this new bottleneck in sample preparation [23–29].

Finally, permanent quality control is necessary to guarantee valid results of this highly sophisticated analytical technique. All working processes in the lab must be well organized, its staff well prepared to face the high demands by sample and data load. In addition, a clearly defined data flow with on-line data transfer, starting with the sample entering the lab, during the complete analysis, and finishing with the analytical result released for the pharmacokinetic evaluation, is indispensable for success.

It is also worthwhile to mention that ‘conventional assays’ using GC or HPLC with UV- or fluorometric detection need not necessarily be replaced by LC-MS/MS procedures especially when being sufficiently sensitive and competitive in sample throughput. Although experience tells that LOQs in the range of 0.1 μg per litre plasma seem to be attainable quite straightforward by LC-MS/MS, sensitivity may also end up to be rather poor, dependent on ionization efficiency or collision-induced fragmentation characteristics of the analyte. In addition, long-term ruggedness of LC-MS/MS compared to HPLC-UV, for instance, is still inferior due to the complexity of equipment. Moreover, trouble-shooting can become quite tricky when not following a strictly systematic approach and performed by experienced, well-trained operators. Finally, investment and maintenance costs for LC-MS/MS equipment are still preventing interested analysts to collect experience with this new bioanalytical tool.

The examples given as well as an increasing number of publications in this area [30–42], however, confirm that these efforts are worth undertaking and also reflect the steadily increasing prominence LC-MS/MS plays in bioanalytics for pharmacokinetic investigations. Recent developments such as the use of LC-MS/MS in simultaneous pharmacokinetic screening of drug candidate mixtures in one animal [43–47] or the growing influence as analytical tool in related areas such as residue and environmental analysis [48, 49] or in the context of combinatorial chemistry [50] further support this optimistic appraisal.

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