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Liquid chromatographic–tandem mass spectrometric method for the analysis of a neurokinin-1 antagonist and its metabolite using automated solid-phase sample preparation and automated data handling and reporting

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Dedicated to Professor Fred M. McLafferty

Abstract

Pharmacokinetic studies play a vital role during the development of new pharmaceutical substances. Data presented demonstrate an accurate, precise and robust assay for a neurokinin-1 receptor antagonist and its metabolite with HPLC-MS-MS. Sample preparation is performed by solid-phase extraction (SPE) in the 96-well plate format. This process is fully automated with a Tecan Genesis™ pipetting system using its standard robotic manipulator arm (ROMA). All instruments are fully integrated in a study oriented laboratory information system (LIMS) with an Oracle database that communicates bi-directional with the analytical equipment. Finally, the results are reported by push button operation.

Keywords: Neurokinin-1 receptor antagonist

1. Introduction

High pressure liquid chromatography coupled to tandem mass spectrometry (HPLC-MS-MS) was a quantum leap in bioanalytical studies, permitting to cope with the increased demands of drug development: More samples, shorter time lines and increased sensitivity due to more potent drugs. Its specificity allows short chromatographic run times of 1–10 mm. As a consequence, sample preparation became a rate limiting step in bioanalysis.

The introduction of solid-phase extraction (SPE) in the 96-well plate format was an advance in high throughput sample preparation [1,2]. This SPE-technique was semi-automated on 4 to 96 tip pipetting devices [2–4] or fully automated by means of a custom built robotic system [5,6].

We describe the use of a 8 tip pipetting device (Tecan Genesis™) using its standard robotic man-

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ipulator arm (ROMA) for full automation of SPE in the 96-well plate format.

An automated method for the quantification of a new drug and its metabolite in the class of NK-1 receptor antagonists [7] was validated according to international guidelines [8] and was successfully applied to the analysis of some 1000 samples from clinical trials.[1]

GLP and GCP requirements [9] have forced a good documentation of all steps performed during a study (protocol of study and its changes, data collection, data handling, exclusion of results). Often, it is difficult to maintain a certain GLP standard during daily work. Delegating this part to a Laboratory Information and Management System (LIMS) improves the GLP standard. The KINLIMS software [10] with the Oracle database is the centre of our study driven activities.

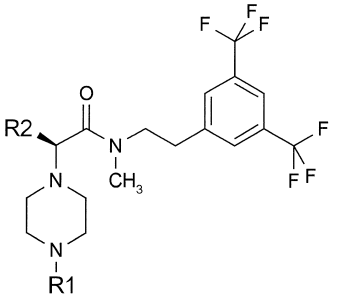
2. Material and methods

2.1. Chemicals and reagents

The reference substances (Fig. 1) were obtained from Boehringer Ingelheim Pharma KG, Biberach, Germany. The stable isotope labelled internal standards were synthesised by the Isotope Chemistry, Department of Pharmacokinetics and Drug Metabolism, Boehringer Ingelheim Pharma KG, Biberach, Germany. Reference substances and internal standards had a certified purity of >97.5%. The purity of all other chemicals and reagents was of analytical grade or better. The drug and its metabolite were enantiomers, the deuterated internal standards however racemates.

2.2. Solutions

All calibration standards, quality control samples and blank samples as well as samples for recovery experiments and stability testing were prepared in human plasma and human urine. Internal standards were solubilised in 50 m*M* phosphate buffer pH 7.0/acetonitrile, 80:20 (v/v) for the addition to plasma (urine).



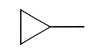
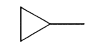
	R1	R2
Drug		C_6H_5
ISTD drug (racemate)		C_6D_5
Metabolite	H	C_6H_5
ISTD metabolite (racemate)	H	C_6D_5

Fig. 1. Structures of the NK-1 antagonist, its metabolite and the corresponding internal standards.

2.3. Instrumentation

Eppendorf centrifuge type 5810 R: Eppendorf, Engelsdorf, Germany; Pipetting robot Tecan Genesis™ RSP 200: Hombrechtikon, Switzerland; autosampler HTS PAL: CTC Analytics, Zwingen, Switzerland; HP 1090 ternary gradient pump: Hewlett-Packard, Waldbronn, Germany; Quattro LC mass spectrometer with Z-Spray™ ion source and MassLynx™ software version 3.2: Micromass, Altrincham, UK.

2.4. Description of the Genesis™ RSP 200

The Genesis™ RSP 200 is a programmable liquid handler, equipped with 8 disposable tips, a robotic manipulator arm (ROMA) and a SPE vacuum station.

All steps of the sample preparation procedure are controlled by Gemini software version 3.00 SP 1. The working list for the sample batch is created by

[1]Parts of this were presented as a poster at the International Symposium on Laboratory Automation & Robotics (ISLAR), Boston MA, 17–20 October 1999.

conversion of a worksheet generated from our Laboratory Information and Management System (KIN-LIMS version 3.0 and 4.0, DataSign AG fuer Informatik and F. Hoffmann–La Roche Ltd, Basle, Switzerland).

2.5. Solid phase extraction procedure

Thawed *plasma* samples were vortexed and centrifuged at room temperature (Eppendorf centrifuge, short spin). The sample vials were put into the Genesis™ RSP 200 for transfer into the 96-well plate format and clean up by automated SPE following the procedure below (pressures and times are adopted to fit to the flow characteristics of various extraction plate lots):

Pipetting: 530 μl internal standard solution (containing 100 ng/ml of internal standards of the drug and its metabolite as well, and 530 μl sample are mixed in a 2 ml 96-well plate.

Extraction plates: Oasis™ HLB 96-well extraction plate (30 mg sorbent).

Conditioning: 1×500 μl methanol, pressure difference 30 mbar, 1 mm; 1×1000 μl 50 m*M* potassium phosphate buffer pH 7.0, 80 mbar, 2 mm.

Sample loading: 1000 μl of the above mentioned sample containing the internal standard; pressure differences: 300 mbar, 6 s; 60 mbar, 3 min; 100 mbar, 3 min; 150 mbar, 3 min; 200 mbar, 3 min; 700 mbar, 1 min.

Washing: 1×1000 μl 50 m*M* potassium phosphate buffer pH 7.0; 1×800 μl 50 m*M* potassium phosphate buffer pH 7.0/MeOH, 1:1 (v/v); 1×200 μl water; pressure difference in each case 150 mbar, 3 min and 700 mbar, 15 s.

The extraction plate was placed on top of a deep well collection plate (1 ml) for collection of the analytes. Elution is performed by 1×150 μl 0.04% conc. formic acid in methanol, pressure difference 30 mbar, 2 min; 1×150 μl 0.04% conc. formic acid in methanol, pressure difference 30 mbar, 2 min; 700 mbar, 2 times 10 s.

Finally 300 μl 50 m*M* ammonium formate buffer pH 4.0 were added to the sample to reduce the elution strength of the solvent.

The well plate was sealed with an aluminium/polypropylene film. The samples were mixed by sonification for 5–10 min and then centrifuged (room temperature, 1000 rpm, about 90 g). The plate was then transferred to the autosampler for analysis.

Urine samples were processed according to the plasma method. During the sample loading the vacuum was slightly lower due to the lower viscosity of this body fluid compared to plasma.

2.6. Chromatographic and mass spectrometric conditions

Injection volume: 120 μl into 100 μl sample loop; column: 70 mm×2.0 mm I.D. +8 mm×3.0 mm guard column, Nucleosil 100-3 C_{18}-HD (Macherey & Nagel, Dueren, Germany); mobile phase: 50 m*M* ammonium formate pH 4.0/MeCN, 45:55 (v/v); flow: 400 μl/min; temperature: 40°C; $t_{r\ (metabo1ite)}$: about 1.4 min; $t_{r\ (drug)}$: about 1.8 min; stop time: 3.5 min.

The flow was split about 1:4 before entering the mass spectrometer.

All spectra and ion chromatograms were recorded on a Quattro LC in the positive electrospray ionisation mode. The spectra of the drug and its metabolite are depicted in Figs. 2 and 3. The deuterated internal standards had the corresponding shifts due to the five deuterium atoms. Source block temperature: 80°C; desolvation temperature: 150°C; nebulising gas flow: about 120 l/h; desolvation gas flow: about 735 1/h; collision gas pressure: about 1.2×10^{-3} mbar; analyser vacuum: about 3.7×10^{-5} mbar; capillary voltage: 3.0 kV; cone voltage: 48 and 53 V (drug and metabolite). Transitions: Drug 528.2 to 229.2 amu, ISTD (drug) 533.3 to 234.2 amu, metabolite 474.2 to 175.2 amu, ISTD (metabolite) 479.2 to 180.2 amu. Dwell times of 0.30 s and an inter channel delay of 0.03 s.

2.7. Validation

Recovery experiments: In order to determine the yield after sample cleanup by SPE in the 96 well plate format human plasma samples containing 0.300 or 30.0 ng/ml drug and metabolite as well were cleaned up following the common procedure, but internal standards (ISTD) were added at different steps of the procedure. For each concentration four samples were cleaned up and ISTD was added after that. The 100% value was determined from four

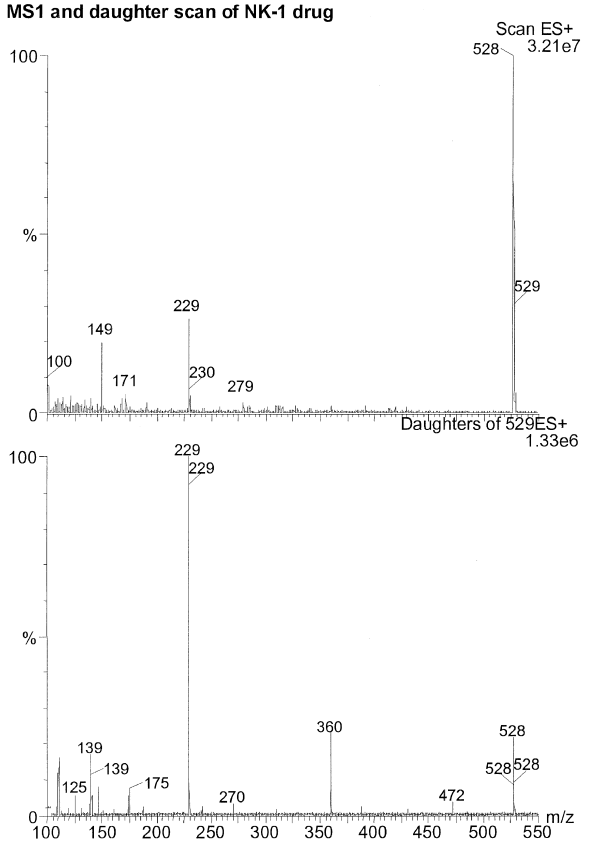


Fig. 2. Full scan electrospray spectrum of the NK-1 antagonist (top, cone voltage 53 V) and daughter scan of 528 m/z (bottom, collision energy 28 V).

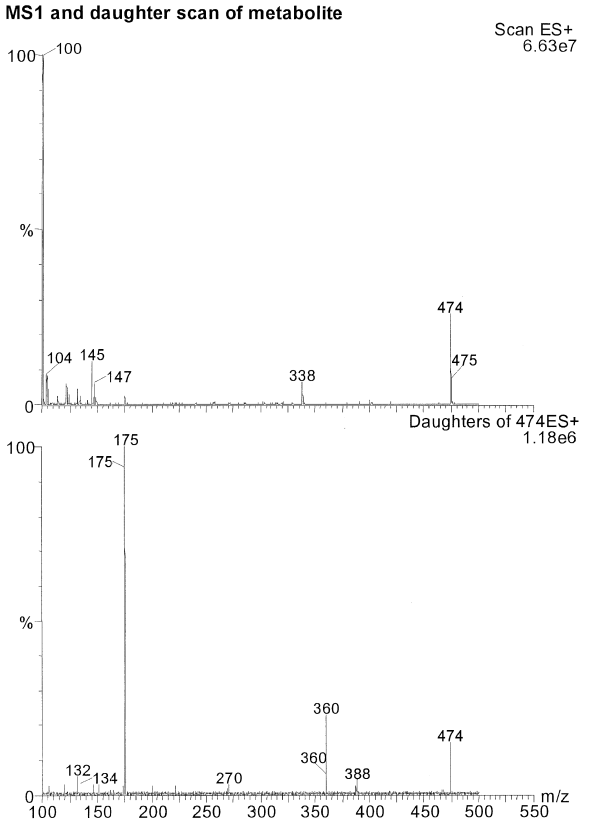


Fig. 3. Full scan electrospray spectrum of the NK-1 antagonist metabolite (top, cone voltage 48 V) and daughter scan of 474 m/z (bottom, collision energy 27 V).

plasma samples with addition of ISTD before SPE. All samples were analysed following the conditions described in Section 2.5. and peak areas were determined.

Accuracy and precision was evaluated as follows: During the validation five *independent* batches were run, each containing 10 calibration concentrations and three sets of quality control samples with concentrations of 0.3 ng/ml, 3.0 ng/ml and 30 ng/ml each in duplicate. Therefore the data of the 0.3, 3.0 and 30 ng/ml are *between batch* results.

Additionally plasma samples with the concentration of the lowest calibration point (0.1 ng/ml) were analysed six fold as unknowns. The values were read on the calibration curve, resulting in the precision and accuracy of these values. The same procedure was applied to samples containing 300 ng/ml which were analysed after a 1:10 dilution.

Therefore, the data for the lowest calibration concentration 0.1 ng/ml and the 300 ng/ml are *within batch* values.

2.8. Automated data handling

KINLIMS and UNICHROM [10] are software packages from Datasign initially developed by F. Hoffmann–La Roche Ltd, Basle, Switzerland, and later co-developed with Boehringer Ingelheim. Both softwares are WINDOWS NT applications and were developed with Uniface as 4th generation tool, C routines for fast calculations are implemented. The Oracle database uses VMS as operating system.

The main objective of UNICHROM is to convert

data from the HPLC-MS/MS into concentration data. This software can handle chromatography, electrophoresis and ELISA data. Additionally, within an analytical technique, different data in different file formats are delivered. To cope with this situation, UNICHROM communicates with programmable import routines and also treats data specifically during data reduction. The calculations of calibration curves and the acceptance testing of batches due to the results of the quality control and calibration samples is a major aspect. This allows an uniform GLP standard for different instruments and facilitates audit trails during data reduction.

Calculated concentration data are transferred from UNICHROM to KINLIMS.

KINLIMS is a study oriented database system in which all possible and relevant parameters of a pharmacokinetic study are reflected in the data model. Its functionality covers all steps during a study: 1) Definition of study (initialisation, study design, sample definition). 2) Execution of study (sample login, data processing, and generation of worksheets and their downloading to the instruments (Tecan Genesis™ and HPLC-MS/MS)). 3) Reporting, data export, closing and archiving of study (Fig. 4).

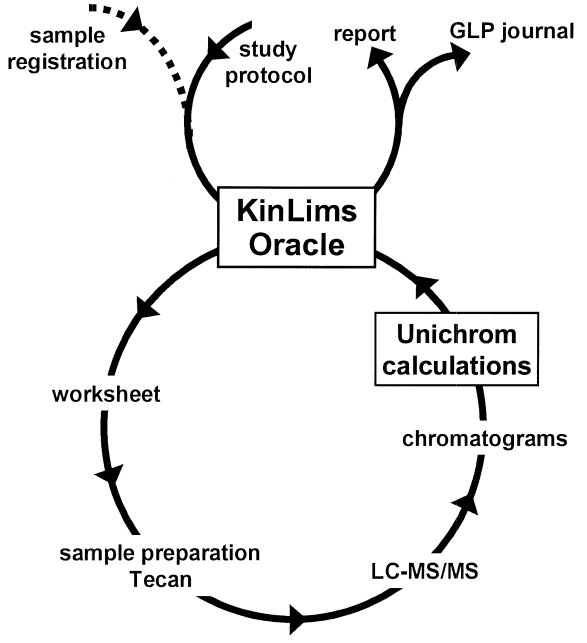


Fig. 4. Integrated data handling: The KINLIMS with the Oracle database is the centre of all study driven activities.

3. Results

3.1. Sample preparation

Processing of two 96-well plates took less than 3 h. There were no causes for manual intervention (e. g. clogged wells) during method validation and analysis of some 1000 samples from clinical trials. To cope with the different flow characteristics of individual wells, a vacuum gradient (increasing vacuum) proved to be very helpful.

3.2. Method parameters

Maximum *carry-over effects* during analysis were below 0.1%.

Specificity: No interference of endogenous compounds was observed in the blank plasma and urine of humans.

Linearity: The calibration curves of undiluted samples were linear over the range of plasma or urine concentrations from 0.100 to 100 ng/ml for drug and metabolite. For plasma the calibration curve for the drug was described by the equation: y (ratio drug/internal standard)=2.324×concentration +0.0306. The correlation coefficient r^2 was in each case of the method validation phase better than 0.9965. For the metabolite the values were: y (ratio metabolite/internal standard)=l.984×concentration +0.0380. The correlation coefficient r^2 was in each case better than 0.9996. For urine the same quality parameter of the regression was obtained.

Chromatograms from plasma are depicted in Fig. 5.

Accuracy and precision was evaluated as described in Section 2.7. According to this procedure, the data of the 0.3, 3.0 and 30 ng/ml are *between batch* results. However, the data for the lowest calibration concentration 0.1 ng/ml and the 300 ng/ml are *within batch* values.

For *plasma accuracy* and precision data obtained in this way are summarised in Table 1. Data during the study are depicted (as an excerpt) in Table 4.

The mean *recovery* from plasma after automated

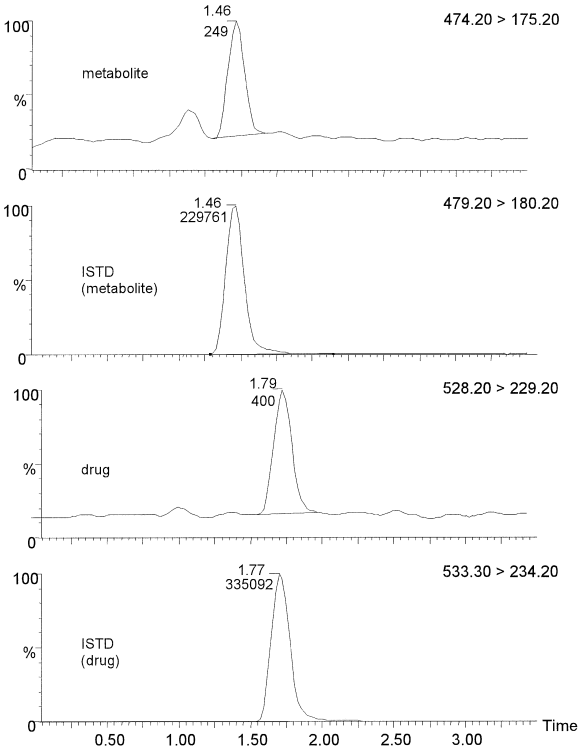


Fig. 5. Ion chromatograms of the lowest calibration sample (C01): blank **plasma** spiked with 100 ng/ml ISTDs (metabolite and drug) each, and 0.100 ng/ml metabolite and drug each.

SPE ranged from 84.4 to 90.9% for the drug (concentration range 0.3–30 ng/ml) and from 75.6 to 86.9% for the metabolite with standard deviations between 1.7 and 5.7% ($N=4$).

Recovery from urine was in the same range for drug and metabolite, too.

Stability: Drug and metabolite were found to be stable in human plasma and urine throughout two thawing–freezing cycles, 24 h at room temperature and about two months at approximately −20°C. In prepared samples the substances were stable for 48 h in the autosampler (room temperature). Accuracy and precision for urine are summarised in Table 2.

3.3. Data handling

The KINLIMS software with the Oracle database is the centre of our study driven activities (Fig. 4). During the initialisation phase, a new kinetic study is defined, which means that all information concerning the study design, involved samples (including calibration standards and QC samples) and analytes to be measured are entered into the LIMS. Using this information, the system is able to calculate the total number of samples and to generate the sample names.

Electronic generation of worksheets represents an important aspect of the KINLIMS: Sample codes are transferred to an empty worksheet, supplemented with vial numbers and dilution factors and then downloaded via local network into the analytical instruments (Tecan Genesis™ and HPLC-MS/MS).

During sample analysis, the worksheet is filled with acquired data (e.g. areas of analyte and internal standard, retention time) and uploaded into the UNICHROM.

Table 1
Accuracy and precision of the quantification method of the NK1 antagonist in plasma

Substance	Concentration [ng/ml]	*N*	Inaccuracy (%)	Imprecision C.V. (%)
Drug	0.100 (LOQ)	6	−9.2[a]	4.3[a]
	0.300	10	−6.5	6.4
	3.00	10	−1.7	4.8
	30.0	10	−3.8	5.5
	300 (dil. 1:10)	6	−6.7[a]	1.2[a]
Metabolite	0.100 (LOQ)	6	−8.3[a]	5.5[a]
	0.300	10	−2.4	5.1
	3.00	10	1.3	2.1
	30.0	10	−1.9	1.9
	300 (dil. 1:10)	6	−3.1[a]	0.8[a]

[a] Measured within one batch.

Table 2
Accuracy and precision of the quantification method of the NK1 antagonist in urine

	Concentration [ng/ml]	*N*	Inaccuracy (%)	Imprecision C.V. (%)
Drug	0.100 (LOQ)	6	−10.5[a]	15.4[a]
	0.300	10	0.9	4.9
	3.00	10	3.4	8.5
	30.0	10	1.9	4.3
	300 (dil. 1:10)	6	−3.2[a]	0.9[a]
Metabolite	0.100 (LOQ)	6	−2.7[a]	9.6[a]
	0.300	10	8.3	4.6
	3.00	10	4.2	8.4
	30.0	10	6.0	5.1
	300 (dil. 1:10)	6	9.4[a]	1.8[a]

[a] Measured within one batch.

In UNICHROM regression analysis of the calibration values is performed and the concentrations of the unknown samples are calculated. The values of the quality control and of the calibration samples are compared with the stored acceptance limits (Table 3) of the calibration curve and the quality control samples. Each deviation is flagged by a warning, if the warning is overwritten we have to comment it in the audit trail.

These concentrations are sent back to KINLIMS with its flexible reporting systems: Summary tables, Excel files and complete analytical reports in user defined, flexible formats. A complete report comprises the acceptance limits, the results of the pre- and main study calibration and quality control samples and the unknown samples. Additionally the audit trail of GLP relevant events is generated.

As an example the list of quality control samples during a study is shown in Table 4. It is interesting to note that the values of the prestudy validation can be set as target values of the main study. These data also demonstrate the quality of the method under routine conditions.

The results of the unknown samples of the study

Table 3
Acceptance limits (and internal control parameters) for sample batches under guidelines of bioanalytical method validation

Acceptance limits		KINLIMS 4.0.22 Prod
Report:	B1125	AR Standard ≠1
Project:	P015/98AF	user name
Protocol:	xyz	GLP
Analyte:	drug [ng/ml]	day-month-year
Method:	HPLC-MS/MS	
	M013/98AF	
Cal. Range:	0.1–100 ng/ml	
Regression Parameter valid as from:	26-MAY-1999	
Limit description:	CHR/GLP 15%	
valid as from:	12-OCT-1994	
Calibration Curve		
Fit Function:	LINEAR	
Weighting:	1/X	
Signal Type:	Peak area	
Internal Standard:	drug-D6 100 [ng/ml]	
Range of calibration curve:	0.100–100 ng/ml	
Extrapolation below lower limit:	0 (%)	
Extrapolation above upper limit:	0(%)	
Range of Calibration Curve:		
Extrapolation below lower Limit:		
Extrapolation above upper Limit:		
Min. valid calibration Points:	8 (N)	
Max. Data Points allowed to be excluded (CLE):	2 (N)	
Max. Calibration Points with warning Flag:	0 (%)	
Max. Deviation between conc. found and conc. added:	15 (%)	
Max. Deviation between conc. found and conc. added at the LOQ:	20 (%)	
Quality Control		
Min. valid QC Samples in a Batch:	4 (N)	
Max. QC Samples with warning Flag:	34(%)	
Max. Deviation allowed from Target concentration:	15 (%)	
Max. Deviation allowed from Target concentration:		
at the lowest QC Concentration (near LOQ):	15 (%)	
Unknown Sample		
Lower limit of quantification	0.100 [ng/ml]	

Table 4
Results of quality control samples (main study phase)

In-Study Quality control Samples					
Report:	B1125				KINLIMS 4.0.22 Prod
Project:	P015/98AF				AR Standard #1
Protocol:	xyz				user name
Analyte:	drug				day-month-year
Species:	man				
Matrix:	plasma EDTA				
Method:	HPLC-MS/MS				
Cal. Range:	0.1–100 ng/ml				
Sample:	**Conc added [ng/ml]**	**Date of Analysis**	**Selected List**	**Conc. found [ng/ml]**	**Dev. (%)**
Q01	0.280	27-MAY-1999	1P270599.AF-2	0.334	19.1
				0.341	21.6
				0.327	16.6
				0.286	2.0
		28-MAY-1999	1P280599.AF-2	0.302	7.7
				0.288	2.7
				0.281	0.2
				0.284	1.3
		10-JUN-1999	1P100699.AF-3	0.296	5.6
				0.290	3.4
				0.291	3.8
		14-JUL-1999	1P140799.MI-2	0.304	8.4
				0.286	2.0
				0.269	−4.1
				0.290	3.4
			1P140799.MI-4	0.291	3.8
				0.283	0.9
				0.264	−5.8
		22-JUL-1999	1P220799.MI-2	0.271	−3.4
				0.260	−7.3
			1P220799.MI-4	0.299	6.6
				0.278	−0.9
		28-JUL-1999	1P280799.MI-2	0.310	10.6
				0.277	−1.2
			N	**24**	
			Mean	**0.292**	**4.0**
			C.V. (%)	**7.0**	
Q02	2.95	27-MAY-1999	1P270599.AF-2	3.18	7.8
				2.96	0.3
				2.96	0.5
				3.11	5.4
		28-JUL-1999	1P280799.MI-2	2.98	1.0
				2.90	−1.7
			N	**24**	
			Mean	**2.90**	**−1.4**
			C.V. (%)	**3.5**	
Q03	28.8	27-MAY-1999	1P270599.AF-2	30.2	4.8
				28.3	−1.8
				29.5	2.2
				28.9	0.2
		28-JUL-1999	1P270599.AF-2	29.1	0.8
				28.6	−1.0
			N	**24**	
			Mean	**28.4**	**−1.7**
			C.V. (%)	**2.6**	

are also reported (data not shown) with two interesting features: If in a batch the lowest calibration point is not valid according to the acceptance limits, but the batch still is valid, automatically the quantification limit of this batch is adapted in the report. Secondly, concentrations, which are the results of reanalysis, are automatically labelled.

4. Discussion

SPE in the 96-well plate format can be fully automated on one workstation using a Genesis™ RSP 200/8 with ROMA and SPE option. The main advantages of this workstation are: Exact vacuum control from 30 mbar to about 700 mbar difference to atmospheric pressure, robotic manipulator arm allowing handling of microtiter plates

Due to the rapid development of automation in all fields of research in the pharmaceutical industry, the adaptation of a standard equipment seems to be advantageous over the development of highly sophisticated systems (which affords often high capacities and time to be implemented).

The analytical method described fulfils the international requirements for bioanalytical assays [8] in all aspects. Accuracy and precision were in the range or even better than for methods where sample preparation includes manual pipetting steps.

With a plasma volume of 500 μl a LOQ of 0.1 ng/ml was attainable which was sufficient to monitor plasma levels in human pharmacokinetics. This sensitivity was obtained without a further concentration (evaporation) step due to the low elution volume. This is a consequence of the relative small bed volume of the extraction phase. The procedure without concentration step is very favourable as it saves time and reduces the risk of losses.

Nowadays, due to the short chromatographic run time of this method and future trends in HPLCMS/MS (multiple inlet technology [11]) and high throughput approaches [12], instrumental capacity is no longer the bottle neck of bioanalytical studies. Sample preparation was subsequently the most time consuming activity. In Fig. 6 the relative time amounts (in man*days) for analysing a human pharmacokinetic study with about 1000 samples is depicted, comparing a solid-phase extraction with a Zymark Rapidtrace™ (three years ago) [13] with the 96-well plate technology, described here. Considering only the cumulated hours for sample preparation a reduction from 7 man*days to two man*days was performed.

Our HPLC-MS-MS method enables the throughput of about 240 unknown samples per day.

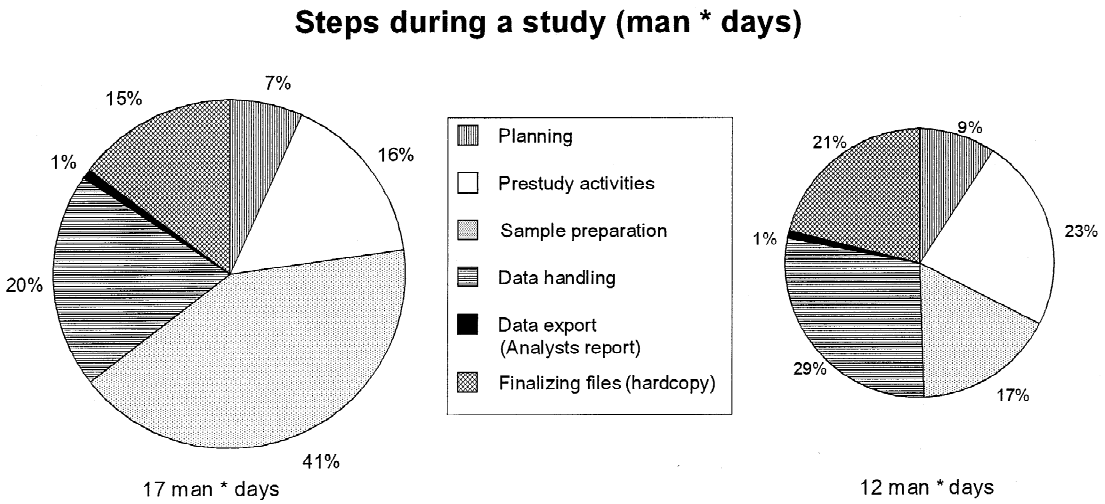


Fig. 6. Relative distribution of manpower (man*days) performing a human study (1000 samples) before and after automated sample preparation in the 96-well plate format.

Planning comprises: Generation of a project in KINLIMS and control of arrived samples. The pre study activities are: Collection of blank plasma, and its analytical control. Calculations and preparations of quality control and calibration samples.

Data handling is rather time consuming. This activity comprises generation of sample lists, control of all chromatograms, UNICHROM calculations of calibration curves, UNICHROM control of batch validity and finally release of data in KINLIMS. Some of these steps with an Oracle based, fully validated system takes more time than a spreadsheet based calculation. But the GLP/GCP environment demands a system which guarantees data integrity and documentation of all activities during a study by an excellent audit trail.

However, automated reporting and data export takes only a low percentage of our workload. In contrast, manual finalizing the hardcopies of the files according to GLP is rather time consuming.

Therefore our future efforts will concentrate on the topics sample registration (by introducing barcodes), and pre study activities. For example, spiking of calibration samples and quality controls is under improvement. Speeding up our data handling by streamlining our UNICHROM activities will bring also a great benefit. A special effort is necessary to finalise the hardcopies for the archive by one push button. Also about an electronic archive we have to think.

5. Conclusion

The fully automated SPE in the 96-well plate format using a Genesis™ pipetting robot showed to be accurate, precise and robust for the analysis of a NK-1 antagonist and its metabolite. Integrated data handling using an Oracle based software facilitates to cope with the GLP/GCP requirements. Permanent improvement of all activities in an analytical lab is necessary to cope with the demand for shorter development times in the pharmaceutical industry.

6. Nomenclature

amu	atomic mass units
NK-1 antagonist	Neurokinin-1 receptor antagonist
GLP	Good Laboratory Practice
GCP	Good Clinical Practice
SPE	Solid phase extraction
ROMA	robotic manipulator arm.

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