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Determination of retigabine and its acetyl metabolite in biological matrices by on-line solid-phase extraction (column switching) liquid chromatography with tandem mass spectrometry

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Abstract

A HPLC assay with tandem mass spectrometric detection in the positive-ion atmospheric pressure chemical ionisation (APCI) mode for the sensitive determination of retigabine [(**I**), D-23129] and its acetyl metabolite [(**II**), ADW 21-360] in plasma was developed, utilising the structural analogue (D-10328), (**III**), as internal standard. Automated on-line solid-phase extraction of diluted plasma samples, based on 200-μl plasma aliquots, at pH 6.5, allowed a reliable quantification of retigabine and the acetyl metabolite down to 1 ng/ml. Injection of 500 μl of diluted plasma onto a C_2 stationary phase-based column switching system in combination with a 75 mm×4 mm reversed-phase analytical column at a flow-rate of 0.5 ml/min provided cycle times of 4 min per sample. The standard curves were linear from 1 to 1000 ng/ml using weighted linear regression analysis ($1/x^2$). The method is accurate (mean accuracy ≤±10%), precise (RSD <±15%) and sensitive, providing lower limits of quantification in plasma of 1 ng/ml for retigabine (**I**), and 2.5 ng/ml for the metabolite (**II**) with limits of detection of 0.5 ng/ml for both analytes. Up to 200 unknowns may be analysed each 24 h per analyst. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Retigabine

1. Introduction

Retigabine [(**I**), D-23129], belongs to a novel class of potent anticonvulsant drugs and is currently being investigated in clinical phase II trials. It is anticipated to act in man against epilepsy, one of the most common neurological disorders with approximately 50 million people affected world wide. Retigabine provided evidence to exert its potent anticonvulsant effects in a broad range of animal models of epileptic seizures after oral administration [1]. The desired anticonvulsant effects could be clearly separated from any adverse neurological side-effects [2,3]. The mode of action generally known for anticonvulsant drugs is based on positive influences on the synthesis of the inhibitory neurotransmitter, GABA, and on opening effects of neuronal potassium channels [4–6]. The acetyl metabolite (**II**) of retigabine, was also found to have anticonvulsant activity but to a lower extent compared to the parent drug [5,7,8]. The metabolite is thought to be formed after cytochrome P450 enzymes and esterases catalysed hydrolysis to the *N*-carbamic acid intermediate followed by enzymatic acetylation [7].

As a result, the development of a sensitive assay which enables the quantification in the lower ng/ml-

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range of both analytes, retigabine (**I**), and its active acetyl metabolite (**II**) had to be envisaged.

Conventional quantitative high-performance liquid chromatography (HPLC) assays with ultraviolet or fluorescence detection for retigabine and the metabolite provided insufficient sensitivity (limit of quantification: 50 ng/ml) and robustness in the lower ng/ml-range. Additionally, these generic methods comprised long chromatographic run times without the use of any internal standards, and labour intensive work-up procedures, which might not be favoured in large clinical pharmacokinetic studies [9].

HPLC with tandem mass spectrometry (MS–MS) using atmospheric pressure chemical ionisation (APCI) in combination with on-line (''column switching'') solid-phase extraction (SPE) should provide the required sensitivity, straightforward sample work-up, and fast capability for trace level quantification of retigabine and its metabolite in biological matrices.

Therapeutic drug monitoring on a ''standby'' basis is often preferred by clinical units for the following reason: dosing can individually adjusted (or terminated) at an early stage of a study in cases of high inter-individual variabilities of bioavailabilities of the drug in combination with undesired side-effects.

Our aim was to develop a fast mass spectrometric method using on-line HPLC–APCI-MS–MS in order to be able to analyse retigabine and its metabolite in human plasma samples in the low ng/ml-range during ongoing pharmacokinetic studies in humans and animals. In order to enable us to report plasma concentrations to the clinical pharmacology unit as fast as possible we envisaged having an efficient sample work-up procedure without time consuming sample concentration/redissolving and sample transfer steps. Therefore, an on-line sample preparation like the ''column-switching'' technique was chosen to be linked with triple quadrupole mass spectrometric detection.

2. Experimental

2.1. Materials

N-[2-Amino-4-(4-flouro-benzylamino)-phenyl]-carbamic acid ethyl ester (retigabine, D-23129) (**I**), *N*-[2-amino-4-(4-flouro-benzylamino)-phenyl]-acetamide (AWD 21-360) (**II**), and the internal standard, 2-amino-3-ethoxycarbonylamino-6-{[(2,4,6-trimethylphenyl)-methyl]amino}-pyridine (D-10328) (**III**), were synthesised within the Department of Chemical Research at ASTA Medica (Frankfurt, Germany). Acetonitrile and methanol were obtained from Merck (Darmstadt, Germany) and were of HPLC-grade. All other reagents and chemicals were of either HPLC- or analytical-grade and were used without any further purification.

2.2. Method

2.2.1. Sample preparation

Blood samples were collected into heparinised or EDTA pre-treated tubes, cooled in ice and centrifuged for plasma (3000 *g*) as soon as possible (<1 h). Plasma samples were kept frozen at approximately -20°C. Frozen samples were thawed at room temperature prior analysis.

To 200 μl of plasma, 100 ng internal standard in 50 μl of 60% (v/v) aqueous acetonitrile and 750 μl of 10% (v/v) aqueous acetonitrile were added to each vial, followed by brief vortex mixing. On-line SPE (SPE ''column-switching'') was carried out employing a 10-port valve system (VICI, Schenkon, Switzerland) feeding two 10×4 mm laboratory-dry packed LiChroprep RP-2 extractions columns with a particle size of 25–40 μm (E. Merck, Darmstadt, Germany). Conditioning of the extraction columns (EC1 and EC2) was accomplished by infusing 10% (v/v) aqueous acetonitrile at a flow-rate of 0.8 ml/min. Volumes of 500 μl from the diluted plasma samples were injected onto the HPLC–MS system.

2.2.2. HPLC–APCI-MS–MS

HPLC was performed on a LiChrospher 60, RP-Select B, 5 μm, 75 mm×4 mm analytical column with 4 mm×4 mm guard columns containing the same stationary phase (E. Merck) using a Consta Metric 4100 MS and a Consta Metric 3200 HPLC pump (TSP, Darmstadt, Germany) and HTS Prep and Load autosampler (CTC, Chromtech, Idstein/Ts., Germany). The injection volume was 500 μl onto the column. The mobile phase consisted of acetonitrile–2.25 m*M* ammonium acetate (pH 6.0) (55:45, v/v) and the flow-rate was 0.5 ml/min.

Mass spectrometric detection was carried out using a Finnigan TSQ 7000 triple quadrupole instrument (Finnigan, San Jose, CA, USA) operating in the positive APCI mode. Selected reaction monitoring (SRM) was employed using xenon (4.0 grade) (Messer Griesheim, Krefeld, Germany) as collision gas at a pressure of approximately 1 mTorr (1 Torr=133.322 Pa), with a collision energy of 15 eV. The APCI-MS operating parameters can be summarised as follows: vaporiser temperature 400°C, sheath gas (nitrogen, 99.99% purity) at 50 p.s.i. (1 p.s.i.= 6894.76 Pa), auxiliary gas (nitrogen, 99.99% purity) flow set at 10 units (exact flow-rates in l/min were not measured), discharge needle at 5 μA, heated capillary temperature at 195°C. The capillary voltage was set at 76.2 V and the tube lens voltage at 104.6 V. Precursor to product ion transitions were monitored at m/z 304 to m/z 230 for retigabine, and at m/z 274 to m/z 256 for the metabolite. For the internal standard the precursor to product ion transitions were monitored at m/z 329 to m/z 133 (Fig. 1). Each ion is monitored for 0.5 s giving a scan time of 1.5 s per cycle. Data were acquired by the Finnigan software (ICIS 8.3.0) and the peak areas measured using the QuanGuide software (ICIS 8.3.0). The mass spectrometer was shut down after overnight runs by instrument control language (icl) procedures.

2.2.3. Preparation of calibration curve

Calibration samples and quality control (QC) samples were prepared with each batch of unknown test samples to cover the range of 1 to 1000 ng/ml. To 200 μl of blank human plasma 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100 and 200 ng of analytes (retigabine and metabolite) in combined standard dilutions of 60% aqueous acetonitrile in volumes of 50 μl were added to give a calibration range from 1 to 1000 ng/ml. The internal standard (100 ng) was added to each tube in volumes of 50 μl giving a final concentration of 500 ng/ml of plasma. After brief vortex mixing, the samples were submitted to automated on-line SPE as described above.

2.2.4. Preparation of quality control samples

QC samples were prepared by an independent analyst at four concentrations covering the range of the calibration curve (QC1: 3 ng/ml; QC2: 15 ng/ml, QC3: 150 ng/ml, and QC4: 750 ng/ml). QC samples were stored for 12 months at −20°C, thawed before analyses, and analysed together with the unknown test samples in every batch.

2.2.5. Quantification

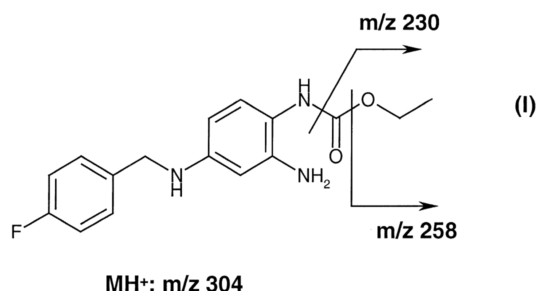
Calibration curves were constructed by plotting peak area ratios of the analytes and the internal standard against the analytes' concentrations. The results of the raw data were transferred to the Finnigan quantification software (QuanGuide, ICIS version 8.3.0). QuanGuide was used to calculate the weighted linear regression fit of the peak areas of the standards of retigabine and the metabolite relative to the internal standard. The weighted ($1/x^2$) linear regression line was fitted over the 1000-fold concentration range. It was found that the weighting by the concentration-squared ($1/x^2$) factor yielded better accuracies for the back-calculated values of the QC samples in comparison to response ($1/y$) or response-squared ($1/y^2$) weighting. Drug and metabolite concentrations in the unknown and quality control samples were calculated from these lines.

3. Results and discussion

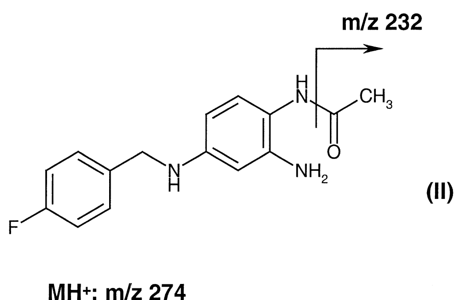
3.1. Mass spectrometry

APCI sources have proven themselves as a very powerful interface between liquid chromatography and triple quadrupole mass spectrometry especially for quantitative biopharmaceutical determinations of moderately polar to non-polar low-molecular-mass (<1500 u) compounds [10]. Interferences from matrix components (e.g., salts and proteins from plasma and urine) appear to have less effects regarding suppression of ionisation of analytes when utilising APCI in comparison to electrospray or pneumatically assisted electrospray (ionspray) [11]. Hence, APCI may be considered as the ionisation method of choice in cases where thermal stability of analytes is guaranteed and high flow-rates of up to 2 ml/min are envisaged.

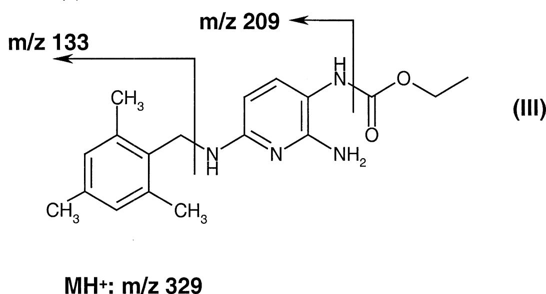
The lack of sufficient sensitivity of a previously developed HPLC assay with fluorescence detection in combination with labour intensive work-up procedures was the driving force for the development of



The major loss of m/z 74 yields the fragment ion used for the quantification of (I) at m/z 230. A minor fragment ion occurs at m/z 258 representing the loss of C_2H_5OH.



The fragment ion at m/z 256 (loss of 18 amu; used for quantification) and the minor fragment at m/z 232 are given for (II).



The fragment ion used for quantification at m/z 133, and the lower abundant ion at m/z 209 are indicated for (III).

Fig. 1. Structures of retigabine (**I**), the major metabolite (**II**), and the internal standard (**III**).

an HPLC–MS–MS assay with a simple, straightforward, and automated on-line SPE.

The deficiency of sufficient selectivity in single MS instruments made it necessary to use a triple-quadrupole mass spectrometric approach. We also envisaged by the application of tandem mass spectrometric detection to minimise the requirements for sample pre-treatment. In order to obtain an extracted plasma sample that is clean enough to enter the mass spectrometer's interface, various off-line and on-line SPE techniques have already proved their suitability in combination with (tandem) mass spectrometric detection in many reported pharmaceutical and bioanalytical applications [12–18].

Two main fragments can be observed by α-cleavages for retigabine (Fig. 1). Fig. 2 shows the APCI

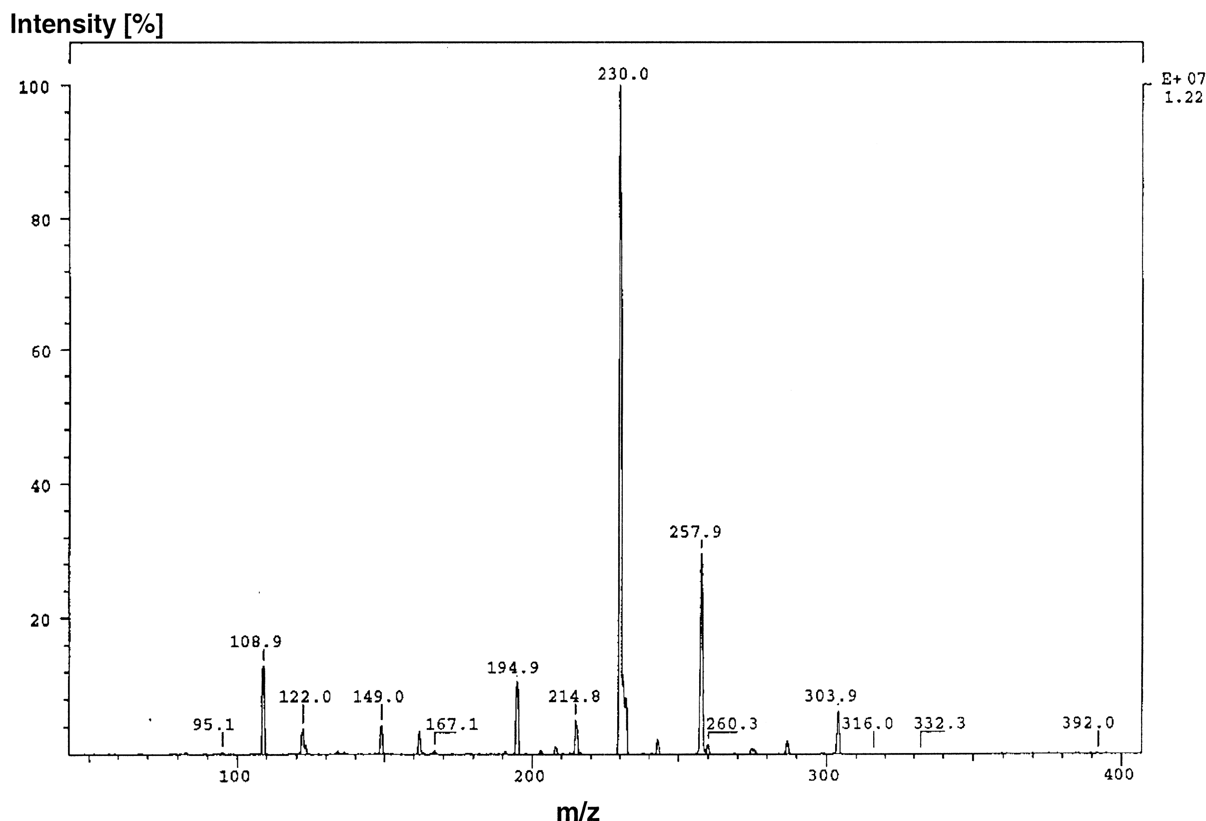


Fig. 2. Positive product ion mass spectrum of retigabine (**I**) precursor ion at *m/z* 304; ~1 ng/μl at a flow-rate of 0.5 ml/min presented to the source (HPLC–APCI-MS–MS mode).

product ion mass spectrum of retigabine using the protonated molecular ion (*m/z* 304) as the precursor ion. The first (minor abundant) fragment ion at *m/z* 258 is produced by a loss of 46 u from the protonated molecular ion representing a cleavage of "ethanol" (C_2H_5OH) from the formic acid ethyl ester group. The most abundant fragment is formed by the cleavage of the amide bond through a neutral loss of 74 u, forming the ion at *m/z* 230, which is used for quantification. The mass spectrometer's parameters (e.g., capillary and lens voltages given in the "tune-file") were set such to optimise the abundance of the major fragment ion at *m/z* 230 of retigabine. In particular, the "up-front fragmentation voltage" [collision-induced dissociation (CID) offset], which can cause general and "less controlable" fragmentation was turned off in order to gain maximum sensitivity for the precursor ions. Thus, an optimum yield of the pseudo-molecular ion (*m/z* 304) of retigabine was allowed to enter the region of the first mass analyser (Q1). As for retigabine, the metabolite shows two major fragment ions derived from the protonated molecular ion at *m/z* 274 (Fig. 1, Fig. 3). In contrast to retigabine under APCI conditions the metabolite shows a base peak at *m/z* 256 which is derived from a loss of H_2O (18 u). This represents the product ion used for the quantification (*m/z* 256) of the metabolite. Another quite abundant ion was observed at *m/z* 232 (with approximately 90% intensity) which might be formed by a loss of the acetyl group bound as amide (loss of 42 u). At these given APCI and MS conditions we discovered two main fragments for the internal standard: a minor fragment ion at *m/z* 209 (abundance of approx. 50%) resulting from two α-cleavages, one at the amide (loss of the formic acid ethyl ester group)

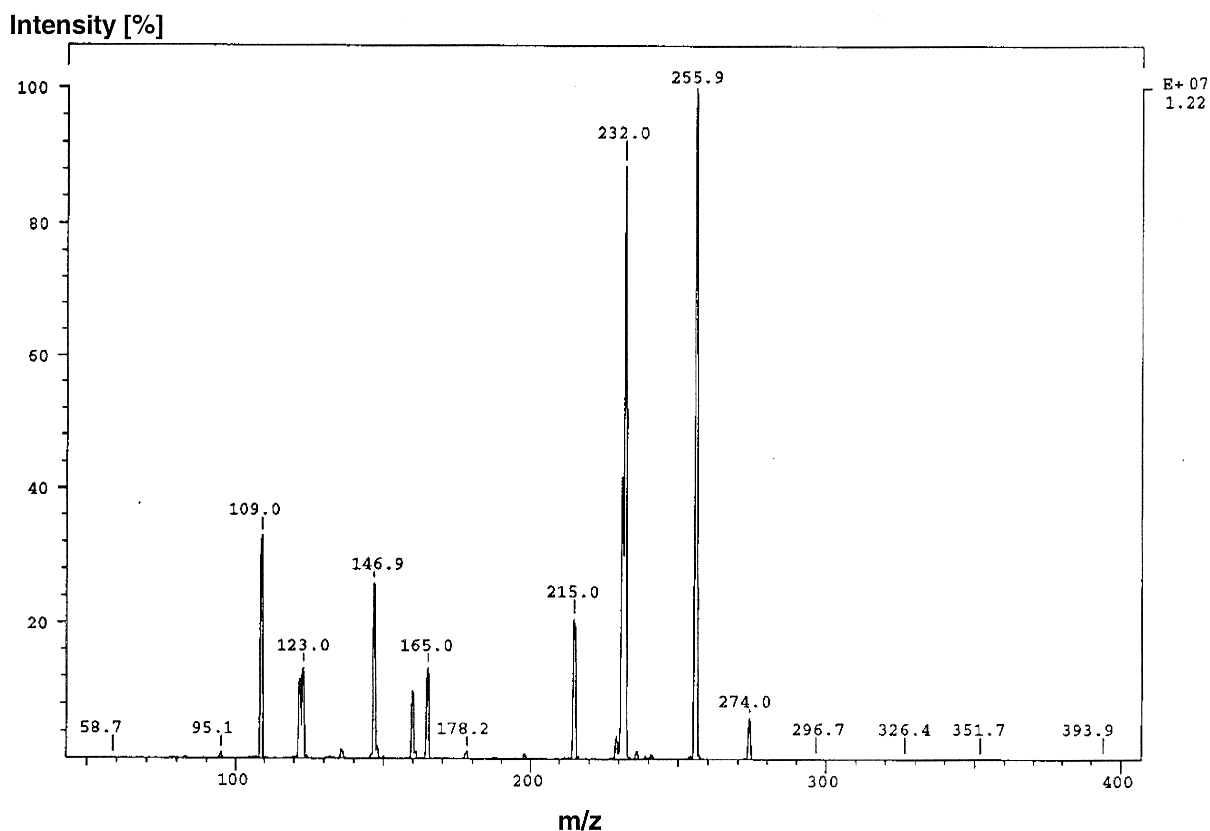


Fig. 3. Positive product ion mass spectrum of the major metabolite (**II**) precursor ion at m/z 274; ~1 ng/μl at a flow-rate of 0.5 ml/min presented to the source (HPLC–APCI-MS–MS mode).

and the other at the 2,4,6-trimethyl-benzyl-amino group forming a 2,3,6-triamino-pyridine derivative. However, the most abundant product ion at m/z 133 which is used for quantification is formed by an α-cleavage at the secondary amine forming a stable 2,4,6-trimethyl-benzyl-amine ion (Figs. 1 and 4).

Our approach in optimising the analytical product ions was to tune the mass spectrometer's parameters for relatively lower mass resolution in the first mass analyser Q1 (Δm_{Q1} of the protonated molecular ion at half peak height was 2 u), and for unit mass resolution in the second mass analyser Q3 (Δm_{Q3} of the product ion at half peak height was approximately 1 u). Hence, a gain in sensitivity was achieved by two factors, first, by turning off the skimmer voltage (CID offset), and thus increasing the yield of protonated molecular ions, and second, by adjusting the resolution in the first mass analyser to approximately 2 Daltons for retigabine and its metabolite. The required selectivity that enabled bioanalysis of retigabine and its metabolite in a complex matrix like plasma was achieved by the collisionally induced dissociation of the protonated molecular ions with xenon as collision gas in the collision cell (Q2: second quadrupole), and subsequently, the setting to unit mass resolution in the third quadrupole region (Q3). Optimisation of the mass spectrometer's parameters regarding the optimum fragmentation and formation of the analytical product ions in order to gain sensitivity was performed for retigabine only. The such obtained MS settings were than subsequently accepted for the mass spectrometric analysis of retigabine and the metabolite in combination with the internal standard in the selected reaction monitoring (SRM) mode. This approach in tuning the mass spectrometer has proved to be suitable for

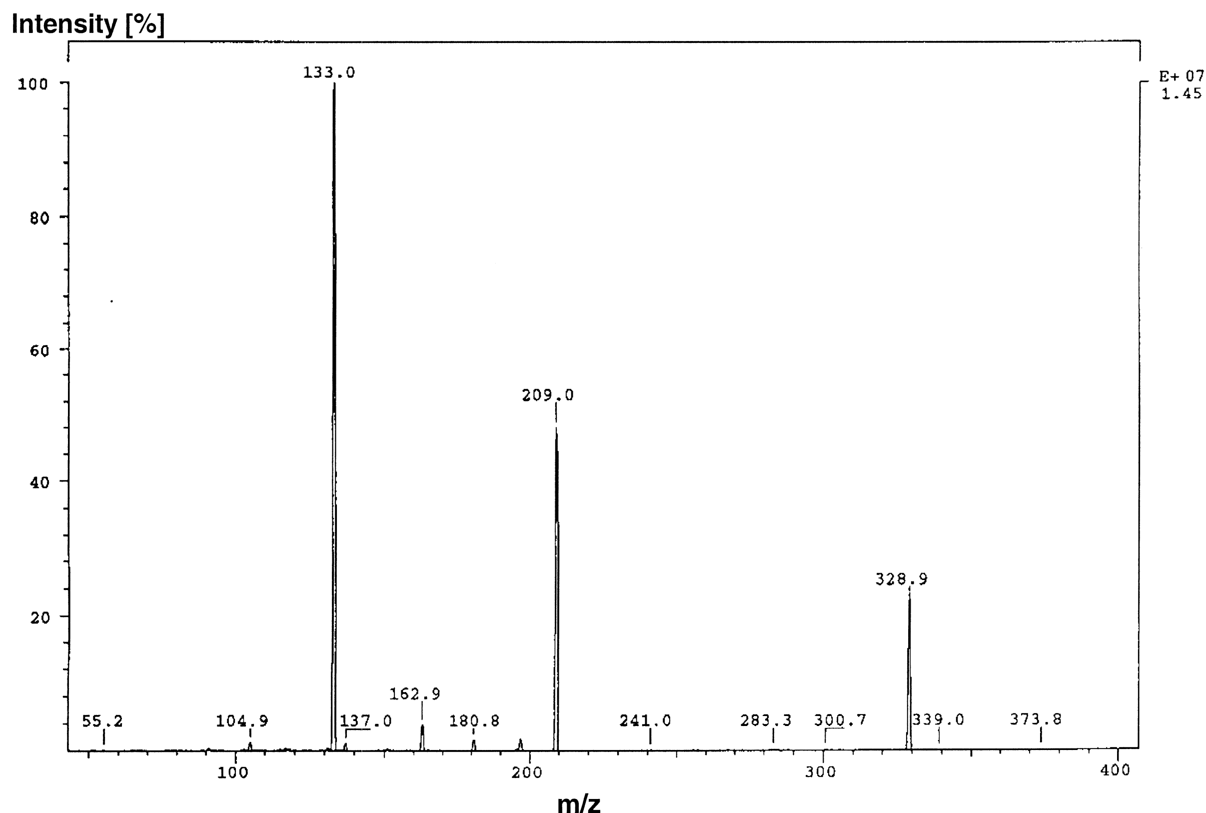


Fig. 4. Positive product ion mass spectrum of the internal standard (**III**) precursor ion at m/z 329; ~1 ng/μl at a flow-rate of 0.5 ml/min presented to the source (HPLC–APCI-MS–MS mode).

the quantification of retigabine and the metabolite since negligible mass "carry-over" from the plasma matrix (i.e., proteins and salts) or the internal standard was observed, operating in the SRM mode. In order to proof this negligible mass "carry-over", a typical product ion chromatogram of a "double blank" plasma sample is depicted and discussed in Section 3.2, illustrating the mass contribution(s) for all three ion-transitions of retigabine [channel (**I**): m/z 304→230], the metabolite [channel (**II**): m/z 274→256], and the internal standard [channel (**III**): m/z 329→133].

3.2. Chromatography

In order to develop an assay with high sample throughput and to obtain maximum response for the analytes we required as short a retention time as possible. The great advantage of having analytes with different molecular masses and similar retention times in combination with tandem mass spectrometric detection, enabled us to aim at a minimum separation that might remove some salts or matrix components that can suppress or interfere with the analyses from the target components, while maintaining good sample throughput. The "chromatographic aim" was to localise the analytes and the internal standard at relatively short retention times without the need to implement long chromatographic run times accompanied with the drawback of decreased sample turnover. Previous experiments have shown that it is paramount to implement at least some crude, minimal chromatographic separation of the analytes for adequate sensitivity and to prevent blockages at the front end of the mass spectrometer, in particular at the heated capillary interfaces and the skimmer region [19]. In this case the prerequisite for a robust routine LC–MS assay can be given by

implementing a relatively "good" chromatographic peak separation of the analytes in combination with APCI. Another important reason why the sample should be separated from some contaminants is that the "cluster-ions" formed in the APCI interface may be depleted when sample and large excess of other components from biological matrices coelute without any guard columns or purification (e.g., SPE). As a consequence proper formation of protonated analytes' molecules could no longer take place, and quantification with reproducible precision and accuracy might not be possible [20]. However, matrix effects in quantitative LC–MS–MS analyses reported by various authors appear to affect the ionisation in the atmospheric pressure chemical ionisation to a significantly lower extent compared to the pneumatically assisted electrospray mode [11].

The persistently rising need for the establishment of high-throughput LC–MS(MS) assays supporting pharmacokinetic in the pharmaceutical industry appears to be the driving force for ultra fast and efficient sample pretreatment techniques such as off-line and increasingly on-line SPE techniques [12–18]. The strong retention of retigabine, the metabolite, and the internal standard even on C_2 reversed-stationary phase material using 10% aqueous acetonitrile as EC mobile phase encouraged us to employ an on-line SPE technique such as "column-switching". Thus, potentially perturbing matrix components (e.g., salts and proteins) are washed into the waste immediately after loading of the diluted plasma samples onto the extraction column already in the first step (Fig. 5a). After valve switching the retained analytes from the extraction columns are submitted to the analytical chromatography by "back-flushing" with the stronger eluting mobile phase (AC eluent: acetonitrile–2.25 m*M* ammonium acetate at pH 6.0, 55:45). During analytical chromatography the second extraction column (EC 2) is loaded simultaneously with the next sample (Fig. 5b). As a result by employing two alternatingly loaded extraction columns during analytical chromatography a very time-efficient sample pretreatment could be set up.

After testing several of the immense variety of chromatographic columns available on the market, we obtained the best results regarding relatively short retention times, and sufficient separation from plasma matrix derived components with the LiChrospher

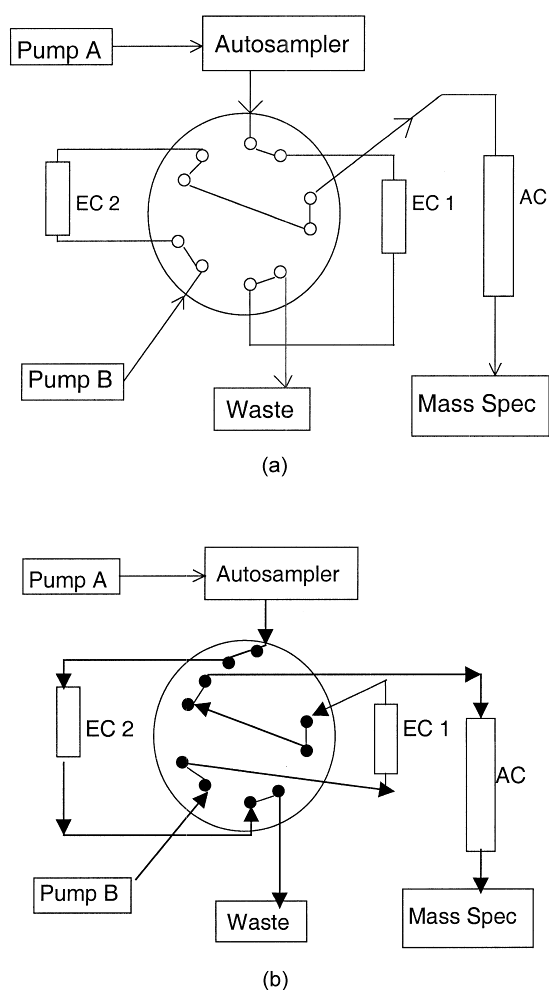


Fig. 5. (a) Step A: loading of sample (analytes) onto EC 1 (extraction column 1) and washing of undesirable matrix components into WASTE (pump A); flow through EC 2 and AC (analytical column) of LC–MS mobile phase (pump B). (b) Step B: elution of analytes from EC 1 (back-flush) onto AC for chromatographic separation and towards the mass spectrometer for detection after valve switching (pump B); simultaneously loading the next sample onto EC 2.

60 RP-Select B, 5 μm, 75 mm×4 mm I.D. stainless steel column (E. Merck). This "reversed-phase" column provided us with the best compromise in terms of reproducibility of chromatographic peak shape, and separation, as well as flow-rate, high sample throughput, and last not least cost effectiveness. All experiments to achieve acceptable chromatographic peaks on other "reversed-phase" col-

umns revealed less satisfactory results . In order to achieve optimum mass spectrometric sensitivity (and fragmentation) for the analytes we decided to keep the LC eluent composition (see Fig. 6 for results of a double blank plasma). Hence, this compromise of sensitivity and chromatographic peak shape was accepted and no further attempts were made to further improve the chromatographic appearance such as ''peak-tailing'' for the analytes at the bottom end of the calibration curve (Fig. 7). The extraction columns (EC 1 and EC 2) were exchanged after the injection of 200 diluted plasma samples (i.e., 100 samples per extraction column). Furthermore, it turned out during routine analysis that one analytical column could easily be used with this set-up of extraction columns for at least 1000 samples without deterioration, such as further increased ''peak-tailing'' and decreasing signals, which we observed after the injection of approximately 1200 on-line extracted (i.e., after ''column-switching'') plasma samples. No further attempts were undertaken to prolong the life-time of these columns, e.g., by washing.

Even replacements of the guard columns offered

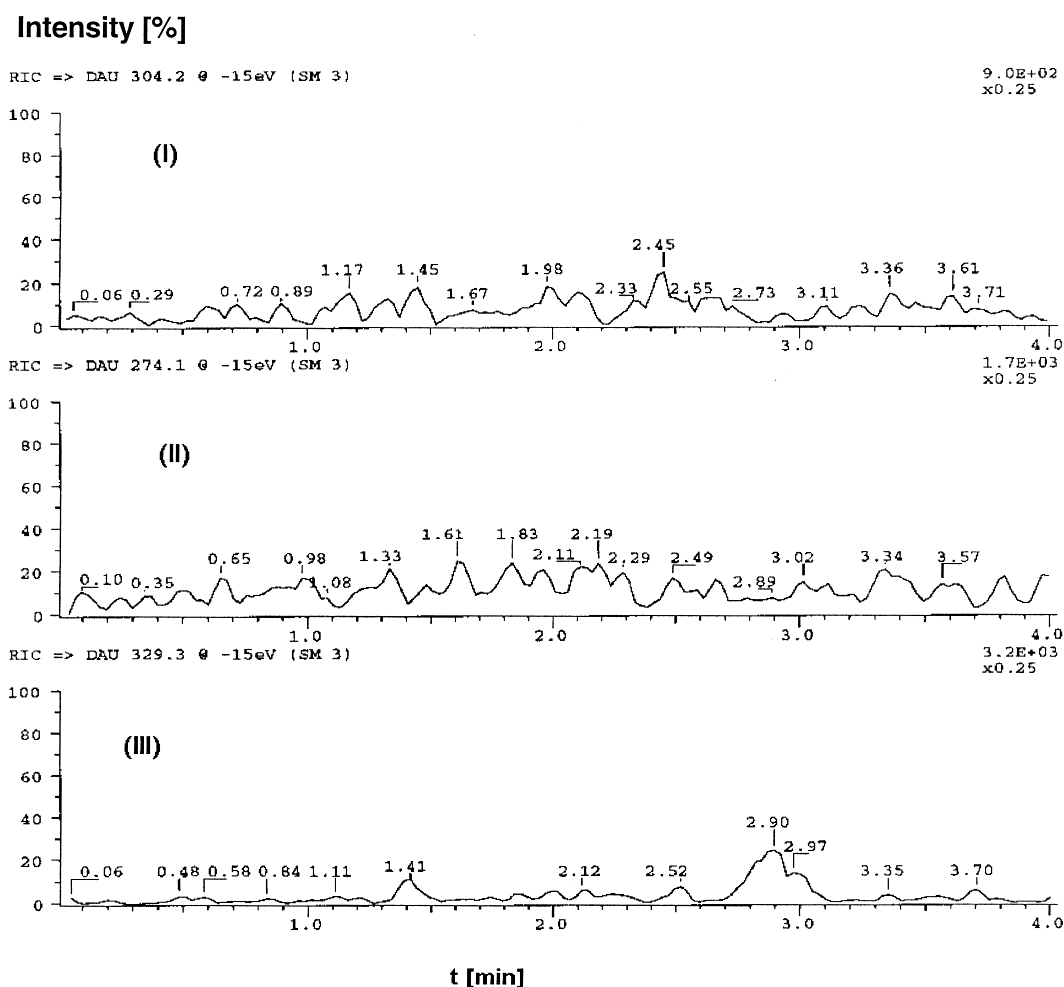


Fig. 6. Typical chromatograms obtained by selected reaction monitoring (SRM) during the determination of retigabine [channel (**I**)], the metabolite [channel (**II**)], and the internal standard [channel (**III**)] in plasma extracts of a ''double blank'' plasma sample. Expected retention times of (**I**), (**II**), and (**III**): 2.11, 1.29, and 3.17 min, respectively.

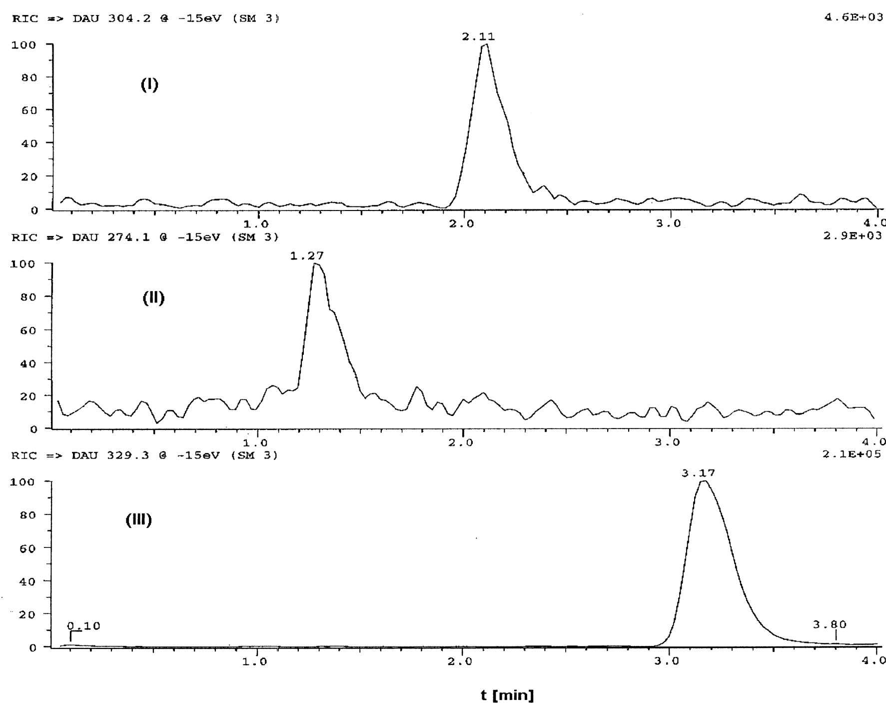


Fig. 7. Typical chromatograms obtained by selected reaction monitoring (SRM) during the determination of retigabine (**I**), the metabolite (**II**), and the internal standard (**III**) in plasma extracts from a calibrator at 2.5 ng/ml, and the internal standard at a concentration of 100 ng/ml.

only marginal improvements of the chromatographic peaks for the analytes. As a result, the analytical columns could not be used for further analyses (i.e., beyond 1200 plasma samples) even after washing with acetonitrile by reversed column flow. However, the observation of such a relatively long life-time of the analytical columns indicates that the on-line-extracted plasma samples were already reasonably clean and this approach suitable for high-throughput routine analysis.

APCI interfaces do not behave as concentration or mass sensitive detectors like the electrospray interfaces [21]. Therefore, a high flow-rate could easily be implemented without any drop in sensitivity as commonly known for electrospray interfaces.

Thus, at a flow-rate of 0.5 ml/min using a 75 mm×4 mm I.D. column and a mobile phase of acetonitrile–2.5 m*M* ammonium acetate (pH 6.0) (55:45) in combination with the APCI interface provided the best compromise regarding mass spectrometric detectability (i.e., sensitivity), chromatographic performance, and analytical ruggedness for the retigabine analysis.

The lack of a suitable stable labelled form of retigabine, forced us to implement another, structurally related derivative as internal standard. The reasons for choosing (**III**) as the internal standard were its similarities in chromatographic and mass spectrometric and chromatographic behaviour to retigabine in terms of the lipophilicity and recovery from biological matrices after on-line SPE on C_2-SPE stationary phase material (recovery of retigabine and its metabolite are approximately 80%).

Owing to the effect of plasma samples enhancing and/or destabilising/stabilising the ionisation process inside the interface of the mass spectrometers and subsequently the signal from the mass analyser, measurements of recoveries against injected stan-

dards may not be reliably achieved. However, recoveries were assessed by analysing blank human plasma samples "spiked" with the analytes prior to the SPE procedure versus standard solutions at the same concentrations injected without SPE. By comparing these results recovery values from the on-line SPE system were estimated to approximately 80% for all analytes. However, these recovery assessments were considered to be satisfactory when simultaneously keeping the advantage of an unattended and automated sample preparation. No precipitation was observed after "spiking" 200 μl of blank plasma with the standard working solutions compared to "spiking" QC or study samples. Therefore, the relatively high percentage of organic "spike" (30% acetonitrile) did not negatively affect the recoveries of the analytes from the matrix. Recovery experiments after on-line and off-line SPE were performed used C_{14}-labelled retigabine showing recoveries ≥80% (results not presented).

In order to illustrate the high selectivity of tandem mass spectrometric detection (SRM mode) selected ion chromatograms of a "double blank" plasma sample "spiked" only with the EC eluent are given in Fig. 6. As a result, negligible contributions occur at the retention times of retigabine, the acetyl metabolite, and the internal standard. Selected ion chromatograms of a plasma extract from a blood sample taken 1.5 h after oral administration of 100 mg of retigabine to a patient, are shown in Fig. 8. At these and even at higher concentrations (>1000 ng/ml) of retigabine (**I**) and the metabolite (**II**) in study samples we observed no matrix related peaks at retention times of 1.29, 2.11, and 3.17 min at the channels of retigabine (m/z 304→230), the metabolite (m/z 274→256), and the internal standard (m/z

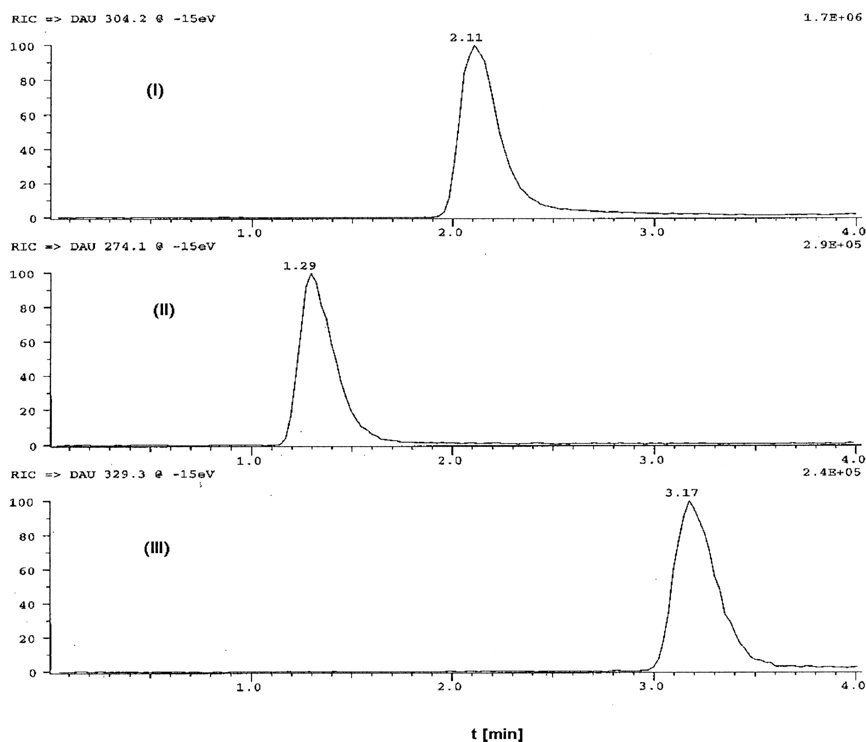


Fig. 8. Typical chromatograms obtained by selected reaction monitoring (SRM) during the determination of retigabine (**I**), the metabolite (**II**), and the internal standard (**III**) in plasma extracts of a blood sample taken from a patient approx. 1.5 h after oral administration of 100 mg retigabine.

329→133). Typical chromatograms of a standard at the limit of quantification of the metabolite (LOQ: 2.5 ng/ml) are presented, revealing reproducible peaks for the analytes, retigabine and the metabolite (Fig. 7) with acceptable assay performances for routine analyses in biological matrices (see Tables 1 and 2).

Retigabine, the acetyl metabolite, and the internal standard eluted at retention times of 2.11, 1.29 and 3.17 min, respectively. The cycle time per sample was 4 min including on-line SPE. The great advantage of a three-step containing on-line SPE by alternating loading and back-flush elution from two extraction columns (EC 1 and EC 2) is outlined in Fig. 5a and b using a 10-port valve for ‘‘column-switching’’.

The detector response for retigabine and the metabolite was linear over the range from 1 to 1000 ng/ml. The limit of detection for retigabine was assessed after injection of 10 standard samples at concentrations near the lower limit of quantification (LLOQ=1 ng/ml) and subsequent extrapolation to a signal-to-noise ratio of $S/N=3$. Accordingly, a limit of detection of approximately 0.5 ng/ml was calculated. In the same manner the lower limit of detection for the metabolite was calculated resulting in a value of 0.5 ng/ml. However, the limit of quantification for the metabolite was to set to 2.5 ng/ml owing to interferences at the 1 ng/ml calibrator.

Typical calibration curves for retigabine comprised a mean slope of 0.0011 [$n=5$; relative standard deviation (RSD)=0.0001] with a mean intercept of −0.0006 (RSD=0.0004) and a correlation coefficient (r^2) of 0.996.

Typical calibration curves for the metabolite comprised a mean slope of 0.0007 ($n=5$; RSD=0.0001) with a mean intercept of 0.0001 (RSD=0.0003) and a correlation coefficient (r^2) of 0.988.

In order to evaluate the inter-assay performance of the analytical method we prepared and analysed standard curves on 5 subsequent days with at least eight QC samples with each standard curve [duplicate QC samples: two towards the lower quartile (3 and 15 ng/ml, the middle (150 ng/ml), and towards the upper quartile (750 ng/ml) of the calibration range]. The intra-assay performance (analysed on 1 day of analysis) was assessed to ensure that the results were acceptable, and could be used for pharmacokinetic analysis. The mean inter-assay precision (RSD) for the standards of retigabine in

Table 1
Inter-assay performance for the determination of retigabine (**I**)

True value (ng/ml)	*n*	Calculated concentration (ng/ml)	Accuracy (%)	RSD (%)
(a) Inter-assay (between-day) performance of retigabine standards				
1	4	1.05	+4.50	2.75
2.5	5	2.24	−10.40	7.87
5	5	4.94	−1.28	6.22
10	5	10.05	+0.50	3.01
25	5	25.30	+1.22	3.48
50	5	48.10	−3.80	4.17
100	5	101.44	+1.44	1.58
250	5	260.05	+4.02	2.79
500	5	507.75	+1.55	4.14
1000	5	1011.56	+1.16	5.25
			Mean accuracy: −0.11	Mean RSD:±4.13
(b) Inter-assay (between-day) performance of QCs				
3	9	3.18	+5.89	4.00
15	10	15.23	+1.56	7.01
150	10	155.41	+3.61	4.17
750	10	786.62	+4.88	6.90
			Mean accuracy: +3.99	Mean RSD: ±5.52

Table 2
Inter-assay performance for the determination of the metabolite (**II**)

True value (ng/ml)	n	Calculated concentration (ng/ml)	Accuracy (%)	RSD (%)
(a) Inter-assay (between-day) performance of metabolite standards				
1	2	1.05	nc	nc
2.5	5	2.37	−5.20	5.52
5	5	5.47	+9.32	8.31
10	5	9.90	−1.03	10.07
25	5	25.09	0.35	7.91
50	5	47.64	−4.73	8.91
100	5	101.43	+1.43	8.88
250	5	250.77	+0.31	8.83
500	5	504.66	0.93	10.16
1000	5	980.94	−1.91	9.11
			Mean accuracy: −0.06	Mean RSD: ±8.63
(b) Inter-assay (between-day) performance of QCs				
3	9	3.03	+0.96	6.50
15	10	16.24	+8.27	10.04
150	10	163.96	+9.31	7.71
750	10	820.96	+9.46	9.15
			Mean accuracy: +7.00	Mean RSD: ±8.35

[a] nc: Not calculated.

plasma was ±4.13% with a mean accuracy of −0.11%, and for the QC samples ±5.52% with a mean accuracy of +3.99% (Table 1). The mean inter-assay precision (RSD) for the standards of the metabolite in plasma was ±8.63% with a mean accuracy of −0.06%, and for the QC samples ±8.35% with a mean accuracy of +7.00% (Table 2). The mean intra-assay precision (RSD) for the QCs of retigabine in plasma was ±5.86% with a mean accuracy of −1.10% (Table 3). The mean intra-assay precision (RSD) for the QCs of the metabolite in plasma was ±8.21% with a mean accuracy of −5.33% (Table 4). Therefore, the overall inter- and intra-assay performances for the standards and QC samples were satisfactory for routine on-line HPLC–MS–MS analysis.

The reported method can also be applied for both, the analysis of retigabine in plasma from other species (i.e., mouse, rat, dog, and monkey) and in urine samples. Achieved assay performances for the analysis of retigabine and the metabolite in plasma matrices of the other mentioned species are identical to assay performances in human plasma (results not shown). All mass spectrometric and chromatographic settings as well as the sample work-up procedure are alike to the retigabine analysis in human plasma.

Table 3
Intra-assay (within-day) performance for the determination of retigabine (**I**) in QCs

True value (ng/ml)	n	Calculated concentration (ng/ml)	Accuracy (%)	RSD (%)
3	4	3.24	7.92	3.83
15	4	14.47	−3.54	12.22
150	4	138.69	−7.55	0.21
750	4	740.40	−1.24	7.17
			Mean accuracy: −1.10	Mean RSD: ±5.86

Table 4
Intra-assay (within-day) performance for the determination of the metabolite (**II**) in QCs

True value (ng/ml)	n	Calculated concentration (ng/ml)	Accuracy (%)	RSD (%)
3	4	3.03	1.01	11.02
15	4	13.43	−10.50	7.43
150	4	138.03	−7.98	6.89
750	4	721.16	−3.85	7.49
			Mean accuracy: −5.33	Mean RSD: ±8.21

4. Conclusion

In conclusion, a fast, sensitive, selective and robust assay with tandem mass spectrometric detection has been developed. The results of the assay performances clearly show that an assay for retigabine and its major metabolite in plasma with a limit of quantification at 1 ng/ml and 2.5 ng/ml, respectively, could easily be established utilising a relatively rough and automated on-line sample clean-up procedure by SPE ("column switching"). This method has been employed successfully for the determination of the pharmacokinetics of the anticonvulsant drug, retigabine and its acetyl metabolite, in preclinical and clinical pharmacokinetic and toxicokinetic studies.

In Fig. 9 an example of a concentration versus time profile is outlined in which a male healthy volunteer received a single dose of 100 mg via oral administration. Concentrations of retigabine (**I**) and the acetyl metabolite (**II**) were measured up to 24 h after administration of retigabine. It could be demonstrated that maximum plasma levels (C_{max}) for retigabine and the metabolite were achieved at approximately 1.5 and 3 h, respectively, after single oral administration of 100 mg of retigabine (within occurring inter-, and intra-individual variabilities in plasma concentrations).

Once more, HPLC–MS–MS in combination with an APCI interface has demonstrated itself to be an excellent tool for fast analysis regarding the support of pharmacokinetic studies of non-volatile, low-molecular-mass compounds especially when lacking sufficient sensitivity and selectivity of conventional HPLC assays with ultraviolet or fluorescence detection.

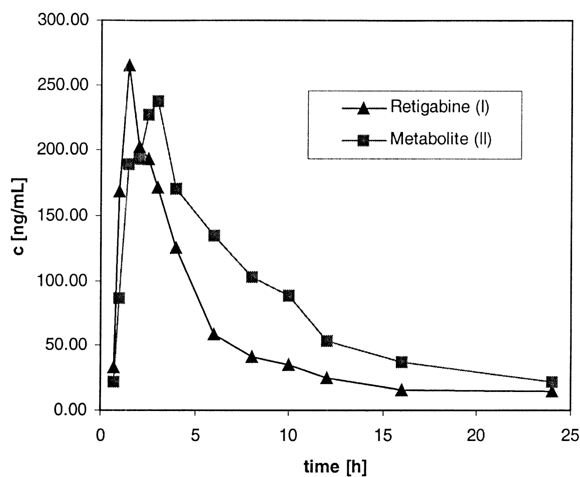


Fig. 9. Plasma concentration versus time profile of retigabine (**I**) and the major metabolite (**II**) after oral administration of a single dose of 100 mg of retigabine to a healthy volunteer.

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