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Analysis of microsomal metabolic stability using high-flow-rate extraction coupled to capillary liquid chromatography–mass spectrometry

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Abstract

A method is described for on-line high-speed extraction of microsomal samples and analysis by capillary liquid chromatography–mass spectrometry (LC–MS) for the determination of metabolic stability in connection with the development of positron emission tomography (PET) tracers. The method allowed direct injections of large sample volumes at a fast extraction rate, providing a gain in both sensitivity and sample preparation time. The calibration curve of the test compound flumazenil (Ro 15–1788) was linear in the concentration range of 1–150 nM, with a correlation coefficient exceeding 0.999. The accuracy of the method ranged from 98 to 101%. A high precision was obtained, with mean intra-assay and inter-assay relative standard deviations of at most 1.4 and 1.5%, respectively, for quality control (QC) samples. The extraction efficiency was determined to be 99.4%, the total recovery 96% and the carryover to $\leq$0.23%. Extractions were performed in a concentration interval of 30–3000 nM without any sign of column overload. The method was successfully used for determining the microsomal metabolic stability of flumazenil. As a result, the described analysis system is currently used for metabolic screening of PET tracer candidates in our laboratory.

Keywords: High-flow-rate extraction; Metabolic stability; Positron emission tomography

1. Introduction

Positron emission tomography (PET) is a valuable tool in the drug development process. The technology, utilising compounds labelled with short-lived radionuclides, mainly ^{11}C and ^{18}F, is sensitive and can be used to obtain reliable data of high precision for non-invasive in vitro and in vivo studies. PET is therefore increasingly utilised in clinical diagnosis and as a means to study drug interactions and receptor functions [1–3]. In connection with the use of PET in drug development, the PET microdosing concept has led to focus on an earlier and faster use of new tracers [4]. Consequently, we are at present developing faster methods for the development of new tracers. Recent significant improvements in synthetic chemistry, especially [^{11}C]carbon monoxide carbonylation reactions, have enabled the rapid synthesis of a large number of labelled compounds [5,6]. These compounds are subsequently biologically screened with respect to body distribution, specific receptor binding and metabolism. Since an extensive metabolism may interfere with PET quantification, it is essential to obtain accurate metabolic information early in the development process. Such information can be acquired with relatively simple in vitro approaches, using for instance liver microsome protocols [7].

The metabolic stability of the radiolabelled tracer can be analysed using high performance liquid chromatography (HPLC) and sensitive on-line or off-line γ-ray detection [8]. Due to the short half-life of PET tracers (e.g. ^{11}C $t_{1/2} = 20.4$ min) the sensitivity is however rapidly decreasing with time. Data obtained from late time points in the metabolic stability assay may therefore be of poor precision and accuracy. A different strategy that has been employed in our laboratory is to analyse the tracer using liquid

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chromatography–mass spectrometry (LC–MS) [9]. In a PET tracer synthesis, only a fraction of the precursor is in fact labelled with the positron emitting radionuclide. This permits analysis of the stable isotope compound using LC–MS, where the sensitivity is, to the most part, constant with time. MS analysis also provides molecular weight information and the possibility to gain structural data from collision induced dissociation. A radiolabelled analyte can however be used as an efficient tool in the development and validation of an analytical method. The extraction efficiency and total recovery can be determined by simple radioactivity measurements of liquids or solids containing the radiolabelled analyte [8,10].

In the analysis of biological samples using LC–MS, sample preparation is needed in order to avoid degradation of the LC column and to minimise matrix effects in the ionisation process [11]. A number of techniques have been designed for increased speed of the sample preparation step. These include off-line approaches such as automated protein precipitation [12] and solid-phase extraction, and on-line techniques using solid-phase extraction [13], restricted-access media [14] and turbulent flow [15–17] or large-particle high flow separations [11,14,18,19]. In turbulent flow chromatography (TFC) [15] high linear velocities (>5 cm/s) are employed together with large-particle sizes (typically 30–50 μm) and large throughpore column frits (≥10 μm) that facilitate the non-retaining passage of sample matrix such as proteins. A high-flow-rate also permits high-speed analysis, where total run times of less than 2 min can be achieved [18,20]. The use of large-particle sizes, however, reduces the chromatographic efficiency. By coupling a large-particle column, operated at a high flow, to an analytical column, under a laminar flow, the separation efficiency can be increased [21–24]. Such a column-switching approach also permits larger injection volumes with subsequent sensitivity gains [25]. Measurements of microsomal metabolic stability and metabolite identification by LC–MS have been performed using TFC [26] and automated or manual protein precipitation, typically with fast LC gradients [27–29].

The objective of the study was to develop a sensitive and accurate method for measuring the microsomal metabolic stability of novel PET compounds using LC–MS. We present a method for on-line high-speed extraction of liver microsome samples, using a linear flow of approx. 15 cm/s, followed by elution onto a capillary analytical column and detection by MS at a reduced flow (approx. 2 mm/s). The method was validated with respect to linearity, precision and accuracy using a test compound, flumazenil (Ro 15–1788), which has previously been used in a number of PET studies labelled with ^{11}C [30,31]. Extraction efficiency, total recovery and carryover could be determined using radiolabelled flumazenil. The utility of the method was demonstrated by the successful determination of the microsomal metabolic stability of the test compound.

2. Experimental

2.1. Materials

Formic acid (p.a.), tris(hydroxymethyl)aminomethane (TRIS) and sucrose (for biochemistry) were obtained from Merck (Darmstadt, Germany). Acetonitrile (Chromasolv) and potassium dihydrogen phosphate were obtained from Riedel de Haën (Seelze, Germany). Potassium chloride and dipotassium hydrogen phosphate (ultrapure bioreagent) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Ethylenediaminetetraacetic acid disodium salt (EDTA) (purum) was obtained from VWR International. D-Glucose 6-phosphate (G6P), glucose 6-phosphate dehydrogenase (G6PDH) and β-nicotineamide adenine dinucleotide phosphate sodium salt (NADP) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a]-[1,4]benzodiazepine-3-carboxylate (flumazenil, Ro 15–1788) (Fig. 1) was obtained from Hoffman-La Roche (Basle, Switzerland). ($^2H_3$)flumazenil and [^{11}C]flumazenil (Fig. 1) were synthesised in-house [32]. Column packing materials Oasis HLB (30 μm particle diameter) and SepPak C_{18} (55–105 μm particle diameter) were purchased from Waters Corp. (Milford, MA, USA) and Kromasil C_{18} (5 μm particle diameter, pore size 100 A) from Phenomenex (CA, USA).

2.2. Column packing

All columns were prepared with a slurry packing procedure, using a Beckman programmable solvent module 126 (Beckman Coulter, CA, USA), packing in fused silica (0.5 mm i.d., 0.75 mm o.d.). A 10% (w/w) slurry was prepared by dissolving the packing material (extraction columns: Oasis HLB and SepPak C_{18}, analytical column: Kromasil C_{18}) in 2-propanol followed by utrasonificating for 10 min. The slurry was transferred to a packing reservoir, to which the fused silica was connected, and a flow of 2-propanol was applied for 30 min at 1.5 ml/min, obtaining a pressure of app. 14 MPa, for Oasis HLB and 2 ml/min ($P \approx 9$ MPa), for SepPak C_{18}. The analytical column was packed under a constant pressure of 30 MPa for 1 h. Extraction columns were cut into 40 mm lengths, whereas the analytical column was 100 mm long. The former were fitted

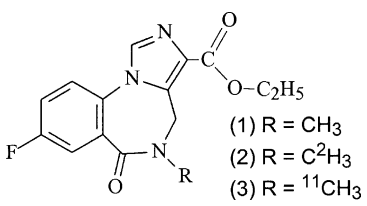

Fig. 1. Structural formulae of analytes. Compound (1) flumazenil (Ro 15–1788), compound (2) internal standard ($^2H_3$)flumazenil, and compound (3) [^{11}C]flumazenil.

with steel screens with a pore size of 10 μm, the latter with a 2 μm pore size, and steel unions.

2.3. Microsome preparation

Freshly excised livers obtained from male Sprague–Dawley rats were rinsed with ice-cold buffer (5 mM TRIS, 1.15% KCl, pH 7.5) in order to remove excess blood and were thereafter scissor-cut on ice and dissolved in the buffer. Homogenisation was carried out with a teflon homogeniser, during which the sample was constantly kept on ice. The homogenate was centrifuged at 9000 × *g* for 20 min at 4 °C. The supernatant was transferred to new tubes and centrifuged at 105,000 × *g* for 60 min at 4 °C. The resulting supernatant was removed and the pellet dissolved in a 10 mM EDTA, 1.15% KCl, pH 7.4 buffer. The microsome solution was once again homogenised and centrifuged at 105,000 × *g* for 60 min at 4 °C. The final pellet was dissolved and finally homogenised in a 10 mM phosphate buffer, 0.25 M sucrose, pH 7.4 and stored at −70 °C for later use. The protein content was determined using the BSA method [33].

2.4. Incubation procedure

The incubation mixtures (500 μl) contained 1 mg/ml microsomal protein, 6 mM $MgCl_2$, 1 mM EDTA, a NADPH-generating system (5 mM G6P, 1 mM NADP and 0.7 IU/ml G6PDH). Dilutions were made with 100 mM phosphate buffer pH 7.4. Before addition of substrate the mixtures were preincubated for 10 min at 37 °C. The incubation was initiated by adding 50 μl of the substrate, dissolved in an aqueous solution containing 0.13% ethanol, and 50 μl of 10 mM NADP to the mixture and placing it in an incubator at 37 °C. The final ethanol concentration in the microsome solution was 0.01%. Reactions were terminated by placing the incubation tube on ice and adding 50 μl of the internal standard ($^2H_3$)flumazenil, for a final concentration of 11.5 nM. Samples were subsequently centrifuged at 143,000 × *g* at 4 °C for 4 min. Two hundred and forty microlitres of the supernatant was transferred to cooled glass HPLC vials (300 μl). In addition, the study comprised control samples with thermally degraded and excluded NADPH-generating systems.

2.5. LC system

A schematic overview of the analytical system is presented in Fig. 2. A Beckman programmable solvent module 126 (Beckman Coulter, CA, USA) was used to load the sample at a flow of 1 ml/min onto the extraction column (40 mm × 0.5 mm, Oasis HLB) using 5 mM formic acid (solvent A). After analyte elution from the extraction column, the same pump was used for a washing step with 95:5 acetonitrile/H_2O in 5 mM formic acid (solvent B). The analytes trapped on the extraction column were eluted onto an analytical column (Kromasil C_{18}, 100 mm × 0.5 mm)

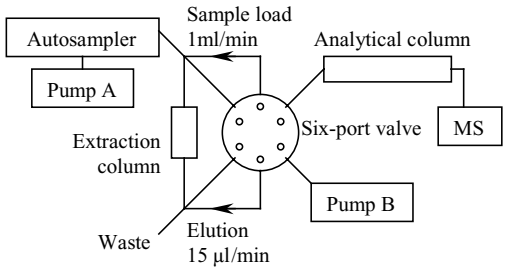

Fig. 2. Schematic representation of the analytical system. Sample loading at 1 ml/min with pump A (Beckman 126) and elution of the extraction column at 15 μl/min with pump B (Shimadzu LC-10AD*VP*).

with a gradient flow of 15 μl/min generated from a Shimadzu LC-10AD*VP* pump system (Shimadzu Corp., Tokyo, Japan), using solvent B and 5:95 acetonitrile/H_2O in 5 mM formic acid (solvent C). The pump was fitted with a 10 μl mixer (Analytical Scientific Instruments, CA, USA), using PEEK tubing (125 μm i.d.) for mixer inlet connections and fused silica (40 μm i.d.) for the outlet. An air pressure actuated six-port valve (VICI Valco Instruments Co. Inc., Houston, USA) was used to direct the flowpaths. A CMA Microdialysis autosampler (CMA Microdialysis AB, Stockholm, Sweden), operated at 4 °C and with a wash solvent consisting of acetonitrile, was used for sample injection. The autosampler was fitted with a 200 μl injection loop, onto which 220 μl of sample was loaded. The CMA software was used to control the valve, pumps and MS acquisition through a contact closure setup. During the loading step, the sample was introduced onto the extraction column at a high flow, focusing the analyte on the column while non-retained sample matrix was diverted to waste. After 0.75 min, the valve was switched and the extraction column was backflushed onto the analytical column with a 4.25 min linear gradient (15–50% B in C). After 4 min, the valve was switched back to the original position and the extraction column and sample loop were washed with a step-wise gradient of 0–100% solvent B. Reequilibration of the extraction column, was initiated at 10 min after the injection, with 100% solvent A. At 11 min the autosampler started the preparation of the next sample, giving a total analysis time of 13 min.

2.6. Mass spectrometry

A VG Platform (Waters, Milford, MA, USA) single quadrupole instrument, operated in a pneumatically assisted positive electrospray ionisation (ESI) mode, was used. The original electrospray needle was refitted with fused silica, as described elsewhere [34], in order to reduce band broadening. The result was a reduction of the internal diameter of the electrospray probe tubing from 100 to 40 μm. The capillary voltage was set to 3.0 kV, the high voltage lens to 0.5 kV and the cone voltage to 30 V. Selected ion recording (SIR) was used for detection of flumazenil at *m/z* 304 and the internal standard ($^2H_3$)flumazenil, at *m/z* 307.

Quantification was performed using the peak area ratios of analyte and internal standard.

2.7. Validation by LC–MS

The linearity of the method was investigated by analysing calibration microsome samples of concentrations 1, 5, 10, 50, 100 and 150 nM flumazenil, with an internal standard concentration of 11.5 nM, in duplicates. A calibration curve was constructed using a weighted regression line, where each point was weighted with $1/s^2$. Concentrations of the calibration standards were recalculated with the obtained equation. The accuracy was determined by calculating the ratio of the measured concentration and the nominal value, multiplied by 100. The precision was investigated by calculating the relative standard deviation (R.S.D.) of measurements of each concentration. The correlation coefficient, R^2, was used to assess the linearity of the calibration curve.

The signal-to-noise ratio (S/N) of the lowest calibration standard was determined using Micromass MassLynx software (Waters, Milford, MA, USA). The S/N was defined as the greatest height of the signal above the mean noise divided by the root mean square deviation from the mean of the noise.

The precision and accuracy of the method was investigated by analysing quality control (QC) microsome samples of low (5 nM), medium (100 nM) and high (150 nM) concentrations, using an internal standard concentration of 11.5 nM. QC samples of 5 and 150 nM flumazenil concentrations were analysed in six replicates on three occasions. The intra-assay precision was determined by calculating the R.S.D. of the six replicates. The inter-assay precision was calculated as the R.S.D. of all 18 samples. A number of 25 QC samples of 100 nM flumazenil was analysed to investigate the precision and accuracy of the medium concentration. The accuracy of the QC samples was determined by dividing the concentration obtained from the weighted regression equation with the nominal value, multiplied by 100.

2.8. Validation by [^{11}C]flumazenil measurements

A crystal scintillation counter was used to measure the amount of radioactivity in solutions containing [^{11}C]flumazenil. The extraction efficiency was determined by comparing the amount of radioactivity collected in the waste fraction with the amount injected onto the extraction column. A total number of 15 columns were investigated on five occasions, using 100 nM [^{11}C]flumazenil dissolved in a microsome solution.

The recovery was determined by measuring the radioactivity of the applied 100 nM [^{11}C]flumazenil microsome sample ($n = 6$), the fraction collected from the tip of the MS probe and the fractions collected in the waste during sample loading and column washing.

To detect possible column overload, radioactivity measurements of the waste fraction were performed. [^{11}C]-flumazenil microsome samples of 30, 300 and 3000 nM concentrations were analysed in duplicates on two occasions.

The carryover was investigated by injecting a blank microsome solution subsequent to a 150 nM [^{11}C]flumazenil sample. The eluate from the MS probe tip was collected from 0 to 11 min from both samples. The carryover could be determined by measuring the radioactivity of the blank sample and dividing the amount with the radioactivity of the 150 nM sample. A number of four samples were analysed.

3. Results and discussion

3.1. High flow extraction

It is known that high flow extraction with large particles in the extraction bed gives a number of advantages for robust sample preparation. The extraction column is less prone to clogging, carryover is reduced and a high speed of extraction can be obtained [21]. In this study, a rapid extraction step (0.75 min) of a large injection volume was enabled by using a high flow through a large-particle extraction column. An optimum in these advantages can be reached when a turbulent flow is generated in the extraction process. The transition from a laminar to turbulent flow in a packed bed, as the flow velocity is increased, is not instantaneous but is rather characterised by a gradual spreading from a few regions of large voids to the whole bed as the flow increases [35]. The onset of turbulent flow in a packed bed is assigned to a Reynolds (*Re*) number of about 5 [36]. The estimated *Re* number from the 0.5 mm i.d. column used in this work was 4.5. The flow characteristics were termed as high flow, since the degree of turbulence could not be verified. Others have designated flows at a similar *Re* number as ultra-high [18].

3.2. MS and calibration curve

A full MS scan (*m*/*z* 50–350) in the positive electrospray ionisation mode was performed by flow injection of flumazenil (Fig. 3). The protonated pseudomolecular ion, $[M+H]^+$, at *m*/*z* 304 was used for subsequent quantification purposes using SIR. A sodium adduct, $[M + Na]^+$, was detected at *m*/*z* 326. The ion at *m*/*z* 258 was most likely the result of in source fragmentation. Fragmentation of flumazenil using atmospheric pressure chemical ionisation has previously been investigated [37]. It was suggested the *m*/*z* 258 fragment was the product of an initial ethyl moiety loss of 28 Da from the ester chain (Fig. 1), followed by an additional loss of water, resulting in an acylium ion (*m*/*z* 258).

The calibration curve displayed good linearity in the investigated concentration range of 1–150 nM flumazenil, with an average correlation coefficient, R^2, that exceeded 0.999. The average slope and intercept were determined to 0.090 ± 0.001 (±S.D.) and -0.001 ± 0.005, respectively. The bias of the calibration standard determination was low (<1.3%),

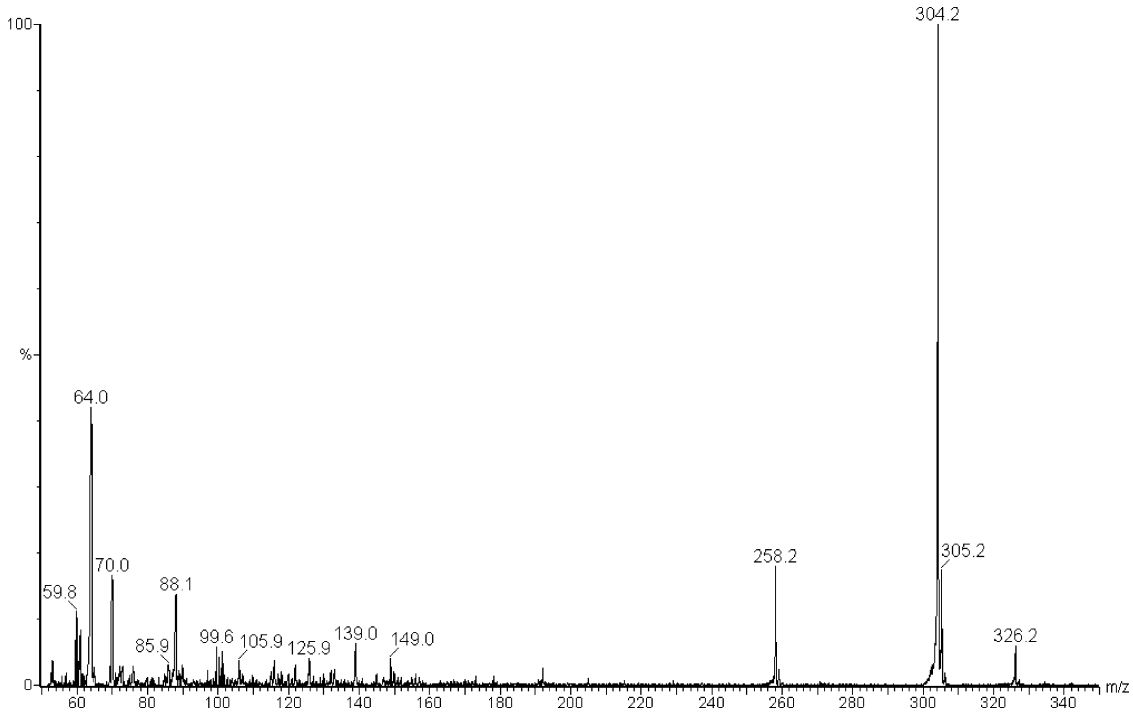

Fig. 3. MS scan (*m*/*z* 50–350) spectrum of flumazenil in positive electrospray ionisation mode.

with an accuracy ranging from 98.9 to 101.3% (Table 1). The inter-assay R.S.D. did not exceed 5.1%, which was the precision established for the lowest calibration standard of 1 nM (Table 1).

Pneumatically assisted ESI-MS behaves as a concentration-dependent detection technique [38] and high sensitivity analysis could thus be promoted by preconcentrating a large sample volume (200 μl) onto a capillary extraction column (0.5 mm i.d.). Subsequent dilution was minimised by using an analytical LC column in the same dimension. The S/N for 1 nM flumazenil microsome samples was determined to 20 ± 2.4 (Fig. 4A).

3.3. Precision, accuracy and specificity

The stability of the developed method was of such magnitude that the accuracy and precision of QC samples could be determined using a calibration curve analysed on one single occasion (Tables 2 and 3). The bias of the method obtained in the analysis of QC samples did not exceed 2.2%. The mean intra-assay R.S.D. was at most 1.4% and the inter-assay

Table 1
Inter-assay precision and accuracy of calibration standards ($n = 4$[a])

Nominal concentration (nM)	Concentration found (±S.D.[b])	R.S.D.[c] (%)	Accuracy (%)
1	1.01 ± 0.05	5.1	101.3
5	5.06 ± 0.09	1.8	101.3
10	10.1 ± 0.18	1.8	101.3
50	50.2 ± 1.31	2.6	100.4
100	98.9 ± 0.14	0.1	98.9
150	149 ± 1.62	1.1	99.3

[a] Data is based on the analysis of two calibration curves, analysing each concentration in duplicates.
[b] S.D., standard deviation.
[c] R.S.D., relative standard deviation.

Table 2
Intra-assay precision and accuracy of QC samples

Nominal concentration (nM)	Concentration found (±S.D.[a])	R.S.D.[b] (%)	Accuracy (%)	*n*
5	4.99 ± 0.05	0.9	99.8	6
5	4.90 ± 0.03	0.6	98.1	6
5	4.92 ± 0.10	2.1	98.5	6
	Mean	1.2		
100	98.3 ± 1.41	1.4	98.3	25
150	148 ± 1.55	1.0	98.9	6
150	148 ± 1.90	1.3	98.7	6
150	147 ± 0.79	0.5	97.8	6
	Mean	1.0		

[a] S.D., standard deviation.
[b] R.S.D., relative standard deviation.

Table 3
Inter-assay precision and accuracy of QC samples

Nominal concentration (nM)	Concentration found (±S.D.[a])	R.S.D.[b] (%)	Accuracy (%)	*n*
5	4.94 ± 0.07	1.5	98.8	18
150	148 ± 1.57	1.1	98.5	18

[a] S.D., standard deviation.
[b] R.S.D., relative standard deviation.

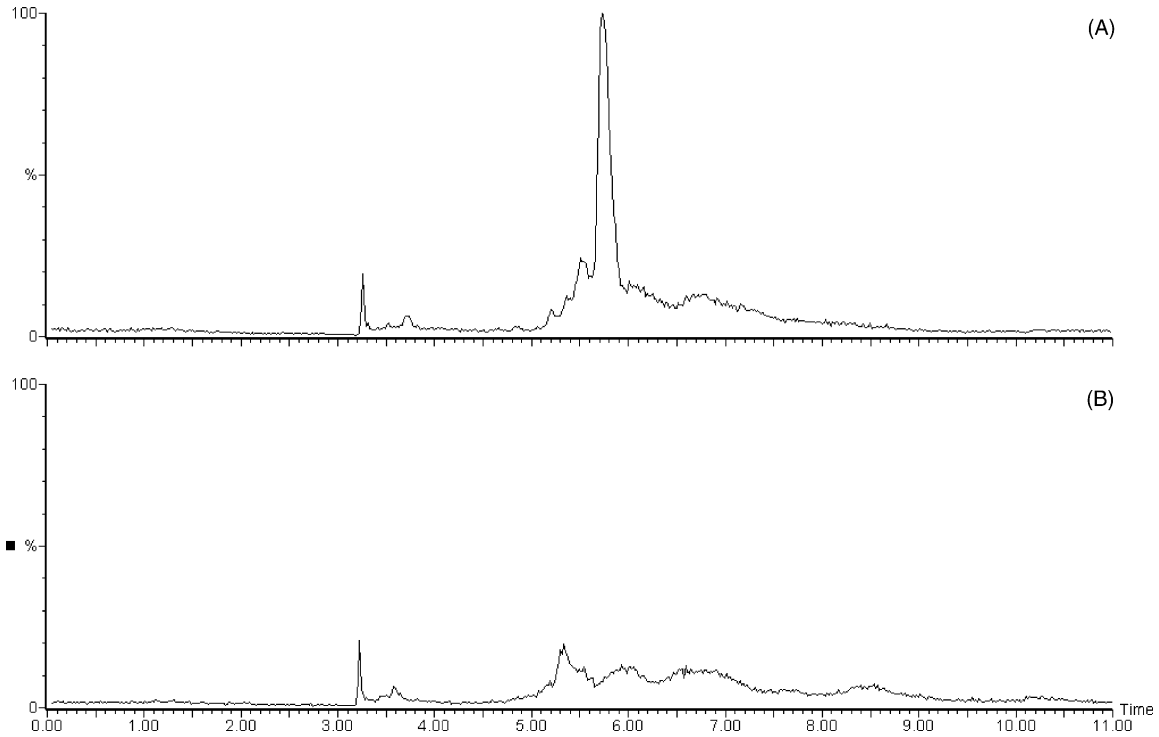

Fig. 4. Selected ion recording (SIR) chromatograms (*m/z* 304) of microsome samples analysed by high flow extraction capillary LC–MS. Injection volume: 200 μl, loading flow: 1 ml/min and elution flow: 15 μl/min. (A) 1 nM flumazenil, S/N = 20 ± 2.4 (±S.D.), and (B) a blank microsome sample.

R.S.D. did not exceed 1.5%. These results indicate that high precision and accuracy analysis can be performed on separate days using low, medium and high analyte concentrations.

In Fig. 4B it can be seen that a blank microsome sample generated a background signal. Utilising a triple quadrupole instrument and multiple reaction monitoring detection would increase selectivity and thus decrease the background signal. However, we consider SIR detection sufficient since the above presented data clearly demonstrate that analysis can be performed with good linearity and very high precision and accuracy utilising the developed method.

3.4. Ion suppression

Ion suppression was measured by comparing peak areas of standards dissolved in water and in microsome solution at a concentration of 50 nM ($n = 5$). Employing Oasis HLB extraction columns the ion suppression was determined to approx. 15%. Interestingly, no ion suppression could be detected when the packing material SepPak C_{18} was used. It was concluded that extraction with the polymer-based material Oasis HLB resulted in a higher degree of co-eluting matrix and therefore a higher degree of ion suppression. This was considered to be a result of the composition of Oasis HLB, with both hydrophilic *N*-vinylpyrrolidone and lipophilic divinylbenzene monomers. The hydrophilic and lipophilic characteristics of the material were however regarded as a positive feature since a generic extraction protocol was sought. Oasis HLB was therefore chosen as the preferred material, even though less ion suppression was obtained utilising SepPak C_{18}. In addition, the packing procedure of SepPak C_{18} often resulted in clogging of the packing system, most likely as a result of the wide particle distribution (55–105 μm).

3.5. Recovery and extraction performance

Determining the absolute extraction efficiency in an on-line extraction LC–MS system can be complicated. Zeng et al. estimated the relative extraction efficiency in a high flow chromatography LC–MS/MS system by using off-line extracted samples analysed by conventional LC–MS/MS as references [22]. Labelling flumazenil with ^{11}C gave us the ability to conveniently establish the extraction efficiency by comparing the amount of radioactivity collected in the waste fraction with the amount injected onto the extraction column. A high extraction efficiency of 99.4 ± 0.1% was thus determined for 100 nM [^{11}C]flumazenil microsome samples. [^{11}C]flumazenil was also used in order to investigate the total recovery of the analyte. 96 ± 1.0% of the amount injected could be collected from the MS probe tip. The fraction collected from the extraction column washing procedure (after the elution step to the analytical column) contained 2.5 ± 0.1%.

An important factor to consider in the extraction process is the concentration of analyte. Increasing the analyte concentration will at some point cause the extraction column to

be overloaded, resulting in reduced extraction efficiency. In order to detect possible column overload at increased concentrations, [^{11}C]flumazenil dissolved in a microsome solution was injected onto the extraction column and the amount of radioactivity in the waste fraction was determined. It was concluded that the extraction performance was stable in the investigated concentration range (30–3000 nM), since no increase of non-retained analyte at higher concentrations could be detected.

3.6. Carryover

We also wanted to investigate possible carryover effects and initially used mass spectrometric detection. However, in the concentration range of 1–150 nM flumazenil the sensitivity of the instrument was insufficient for such determinations. Instead, radionuclide measurements had to be used to estimate the carryover. A blank microsome sample was injected subsequent to a 150 nM ^{11}C-labelled flumazenil sample and the MS probe eluate from both samples was collected. The carryover was estimated to be $0.23 \pm 0.01\%$. This carryover test provided not only a measure of labelled substance eluting at the analyte retention time, but also the total amount of remaining substance from the previous injection. One must also remember that the accuracy of the described carryover test is dependent on the radiochemical purity (>95% throughout this study). It can, however, be concluded that the carryover did not exceed the calculated figure. Furthermore, the carryover was considered acceptable for the method.

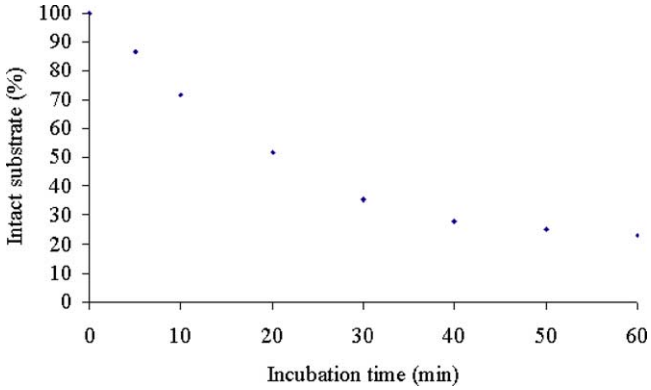

Fig. 5. Determination the microsomal stability of flumazenil. Incubations were performed with an initial concentration of flumazenil of 50 nM and a constant concentration of the internal standard ($^{2}H_3$)flumazenil, of 11.5 nM. Each point represents an average of two LC–MS analyses.

3.7. Application

The described method was applied in the successful determination of the microsomal metabolic stability of flumazenil. After 20 min incubation, 48% of the substance had been metabolised (Fig. 5). The resulting chromatograms from 0 to 60 min incubations showed that 23% of the substrate remained intact in the latter incubation (Fig. 6). In man hepatic metabolism has been reported to be rapid and extensive, with a short elimination half-life of 0.7–1.3 h and a high plasma and blood clearance of 520–1300 ml/min [39]. Each point in Fig. 5 corresponds to a total analysis time of 13 min with a rapid extraction step of 0.75 min.

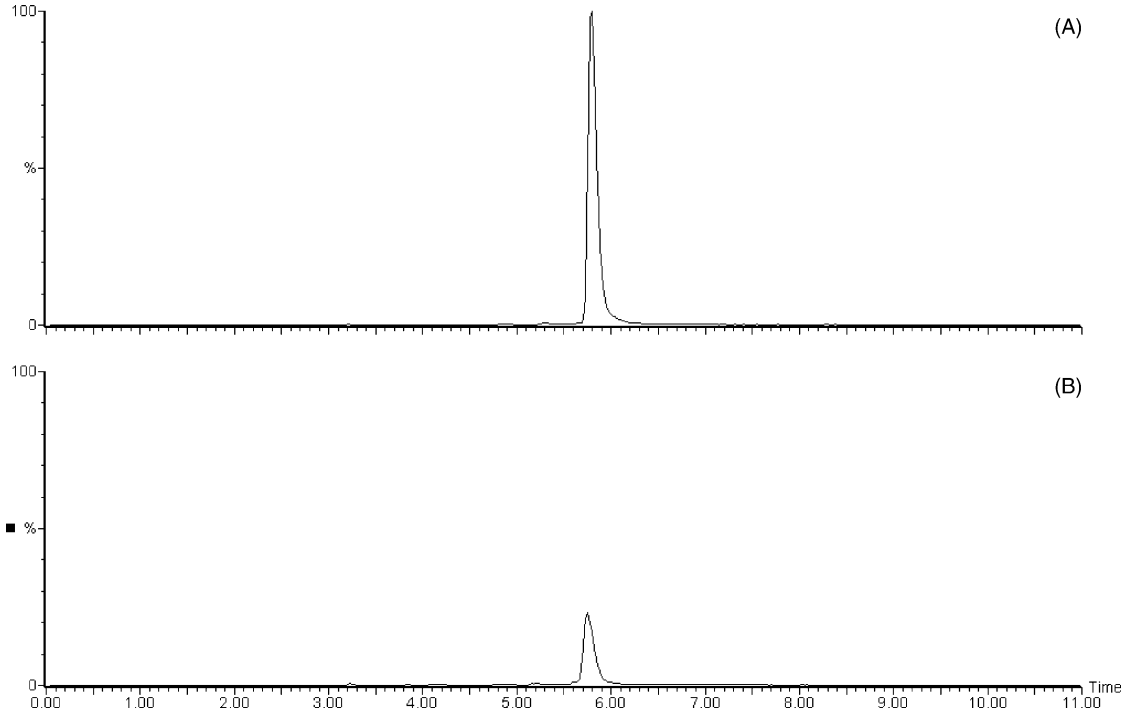

Fig. 6. Selected ion recording (SIR) chromatograms (*m*/*z* 304) from the determination of microsomal stability of flumazenil. (A) 50 nM flumazenil at 0 min incubation and (B) 60 min incubation, 23% of the substrate remained intact.

4. Conclusions

The described method enabled a time efficient and high quality throughput analysis for the determination of metabolic stability. Manual sample handling was minimised by on-line high-speed extraction (0.75 min) that was achieved by directing a high flow through a large-particle extraction bed. Capillary scale columns in combination with a large injection volume promoted high sensitivity ESI-MS analysis that was of high precision and accuracy. The power of using ^{11}C-labelled tracers in the development and validation of an analytical method was demonstrated. A high extraction efficiency and total recovery could thus be determined conveniently. The method was successfully applied in the determination of the microsomal metabolic stability of the test compound flumazenil. We are currently utilising the described analysis system for the determination of the metabolic stability of a number of PET tracer candidates.

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