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# Determination of metabolic stability of positron emission tomography tracers by LC–MS/MS: An example in WAY-100635 and two analogues

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**Abstract**

A method is presented for determination of microsomal metabolic stability of potential positron emission tomography (PET) tracers by LC–MS/MS in the lower nm range. The PET tracers used for the study were the serotonin receptor antagonist WAY-100635 and two potential tracer analogues. The sensitivity permitted the substrates to be directly collected from PET radiolabelling batches, containing very low amounts of substance (0.3–7 μg), for subsequent metabolic stability incubations. Sample preparation was minimal, with addition of internal standard, acetonitrile and a fast centrifugation step, as a result of the low protein concentration of the microsome solutions. Linearity ($R^2 \geq 0.99$), precision (inter-assay R.S.D. < 7%) and accuracy (bias ≤8%) for the tested concentration range 0.5–5 nM proved to be well within accepted limits. No significant differences in metabolic rates were detected using substrates from cold (non-labelling) chemistry syntheses and PET labelling batches, indicating the validity of using substrates from the latter source. A *para*-methoxy-benzamide analogue (MeO-WAY) displayed a significantly lower rate of metabolism compared to WAY-100635, whereas a *para*-iodo-benzamide analogue was more susceptible to metabolic transformation. LC–MS/MS Analysis of formed metabolites from WAY-100635 and MeO-WAY suggested similar metabolic pathways, with hydroxylation, demethylation and dearylation reactions. The main metabolic route in humans, amide hydrolysis, was not observed with the rat liver microsome assay.


*Keywords:* Positron emission tomography; LC–MS; Metabolic stability; Metabolism; WAY-100635

## 1. Introduction

Positron emission tomography (PET) is a sensitive in vivo imaging technique used in biochemistry research, clinical diagnosis and drug development. By labelling compounds with short-lived radionuclides such as $^{11}C$, $^{15}O$, $^{13}N$ and $^{18}F$, quantitative images of, for instance, receptor distribution in the brain can be obtained [1].

In a PET study the concentration of radiotracer in plasma generally needs to be established. Since it is not possible to distinguish between the intact tracer and radiolabelled metabolites using PET, plasma is withdrawn from the subject during a study, for separation and analysis by, for instance, LC and radiodetection [2]. An extensive metabolism, combined with the short half-life of the used radionuclides ($^{11}C$; $T_{1/2}$ = 20 min), can yield samples containing very low amounts of radioactivity at late time points in a PET study. This may yield plasma data of poor precision and accuracy, which in turn may affect the quality of PET data such as receptor binding potential. Additionally, radiolabelled metabolites can potentially enter the target area of the radiotracer, thus complicating PET quantification. It is thus important to investigate the metabolism at an early stage in the development of a new PET tracer.

PET imaging of 5-hydroxytryptamine 1A (serotonin, 5-$HT_{1A}$) receptors [3,4] has been performed with the selective 5-$HT_{1A}$ receptor antagonist WAY-100635 [5,6] (Fig. 1) labelled with $^{11}C$ [7–9]. [carbonyl-$^{11}C$]WAY-100635 is an example of a radiotracer with extensive metabolism [10], with less than 10% of the radioactivity in plasma being the intact tracer 10 min after injection in human volunteers [11,12]. Attempts have been made to develop analogues to WAY-100635 that display a maintained high 5-$HT_{1A}$ selectivity, but with a reduced metabolism [13–17].

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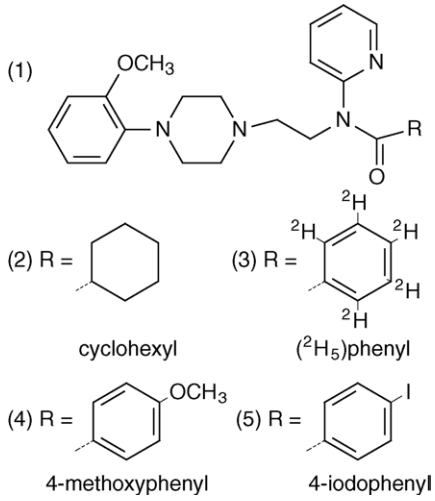



Fig. 1. Structures of WAY-100635 and analogues. (1) Backbone of the compounds, (2) WAY-100635, (3) WAY-$d_5$ (internal standard), (4) MeO-WAY and (5) I-WAY.

We are undertaking research to explore other approaches and to develop new viable $^{11}$C-labelled WAY-100635 analogues [18] in line with the PET microdosing concept for faster development and earlier use of new tracers [19]. A number of WAY-100635 analogues have thus been labelled in the carbonyl position using [$^{11}$C] carbon monoxide [18] for subsequent screening with autoradiography and biodistribution assays. The best candidates of this screening are thereafter assessed in vitro according to their metabolic stability and the top ranked tracers subsequently evaluated using in vivo PET experiments. Sensitive metabolic analysis can be achieved by LC and radiodetection [2,20]. However, as discussed above, the sensitivity is rapidly decreasing with time, which may lead to measurements of low precision and accuracy. Additionally, the short half-life adds restrictions on the number of samples that can be analysed from one labelling experiment.

A different approach for analysing PET tracers in biological matrices has previously been explored, using LC–MS(/MS), whereby the unmodified and stable fraction of the radiotracer is measured [21–23]. The main advantage of LC–MS analysis of the stable isotope compound lies in a sensitivity that is, in principle, not time dependent. Theoretically, this leads to a constant precision and accuracy with time. LC–MS analysis also holds the potential for highly efficient metabolic screening of radiotracer candidates. If high sensitivity is reached, the substrate can be collected from the labelling batch used for biodistribution and autoradiography assays. The labelling batch can be stored and used for repeated metabolic stability assays, which is not possible with radiodetection of short-lived radionuclides. Also, MS analysis provides structural information and high selectivity.

A number of methods utilising quadrupole mass spectrometers have been developed for determination of in vitro metabolic stability of drugs [24–28]. In vitro generated metabolites of fluorinated WAY-100635 analogues have previously been identified with LC–MS/MS and the metabolic rate determined with radioactivity measurements [29,30]. The metabolic profiling was performed with initial substrate concentrations of approximately 45 μM. The amount obtained from $^{11}$C-labelling batches is typically very low [19] (0.3–7 μg or 0.6–17 nmol in this study). If the substrate is to be collected from such a batch, the metabolic stability analysis typically needs to be performed in nM concentrations, which calls for high sensitivity of the analytical method.

The objective of this work was to develop an LC–MS/MS method, with good precision and accuracy, for determination of microsomal metabolic stability of potential PET tracers, in the lower nM range, obtained from a $^{11}$C-tracer production protocol. The radiotracers chosen for the study were WAY-100635 and two analogues. Additionally, the identities of metabolites of WAY-100635 and the analogue displaying the greatest metabolic stability were investigated.

## 2. Experimental

### 2.1. Materials

Formic acid (pro analysis), tris(hydroxymethyl)aminomethane (TRIS) and sucrose 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). Cyclohexanecarboxylic acid {2-[4-(2-methoxy-phenyl)-piperazin-1-yl]-ethyl}-pyridin-2-yl-amide (WAY-100635) (Fig. 1) was purchased as a maleate salt from Sigma–Aldrich. *N*-{2-[4-(2-Methoxy-phenyl)-piperazin-1-yl]-ethyl}-*N*-pyridin-2-yl-($^2$H$_5$)benzamide (WAY-$d_5$) and 4-methoxy-*N*-{2-[4-(2-methoxy-phenyl)-piperazin-1-yl]-ethyl}-*N*-pyridin-2-yl-benzamide (MeO-WAY) were prepared according to previously described methods [13,31]. 4-Iodo-*N*-{2-[4-(2-methoxy-phenyl)-piperazin-1-yl]-ethyl}-*N*-pyridin-2-yl-benzamide (I-WAY) (Fig. 1), WAY-100635 and MeO-WAY were additionally obtained from a $^{11}$C-labelling batch [18].

### 2.2. Incubation procedure and sample preparation

Microsome solutions were prepared as described previously to a concentration of 1 mg/ml microsomal protein and with a NADPH-generating system (5 mM G6P, 1 mM NADP and 0.7 IU/ml G6PDH) [22]. This solution was diluted with 100 mM phosphate buffer (pH 7.4) to obtain a protein concentration of 0.05 mg/ml. Additionally, a solution lacking the NADPH-generating system was used as a control sample. The microsome solution was divided into 1.5 ml Eppendorf tubes for pre-incubation at 37 °C for 10 min. Forty microliters of 78.8 nM WAY-100635 and 40 μl of 78.8 nM of the analogue, each dissolved in water–methanol (95:5, v/v), 5 mM formic acid, and 50 μl of 0.6 mM NADP were added to each tube.

The final methanol concentration in the microsome solution was 0.6% and the substrate concentration 5 nM. Incubations were performed during 0, 5, 10, 15, 20, 30, 40 and 50 min (37 °C). The control sample was incubated for 50 min. For metabolite identification assays, with an incubation time of 2 h, the substrate was added to a concentration of 10 μM for WAY-100635, and 7.5 μM for MeO-WAY (obtained from cold, non-labelling syntheses), with a final methanol concentration of 1.3 and 3% for WAY-100635 and MeO-WAY, respectively. Metabolic reactions were terminated by placing the incubation tube on ice and by adding 168 μl of ice cold acetonitrile, 5 mM formic acid, to give a final acetonitrile concentration of 20%. Apart from aiding in stopping enzymatic reactions, this last step was performed to increase the solubility of the analytes in the chilled Eppendorf tubes, and later in LC vials placed in a cooled (4 °C) autosampler. Forty microliters of the internal standard, WAY-$d_5$, was thereafter added, for a final concentration of 0.8 nM (not performed for metabolite identification assays). Samples were subsequently centrifuged (20,200 × *g*) at 4 °C for 5 min using an Eppendorf 5417R instrument (Eppendorf AG, Hamburg, Germany), in order to remove possible particulate matter. An aliquot of the solution (400 μl) was transferred to cooled glass LC vials (total recovery vials, 1.5 ml, Waters, Milford, MA, USA) for subsequent LC–MS analysis.

### 2.3. LC–MS

A Waters 2695 system (Waters, Milford, MA, USA) was used for pumping the mobile phase, injection of samples and heating of the column. The mobile phase consisted of 10 mM formic acid in water (A) and 10 mM formic acid in acetonitrile (B). The pumps were programmed to deliver a gradient running from 10 to 70% of mobile phase B in A, from 0 to 5 min, at a flow-rate of 0.3 ml/min. The composition was returned to 10% B in A from 5 to 5.5 min. The end time of the programme was set at 10 min. A delay volume of 400 μl was added in order to compensate for the pump dead volume (approximately 0.6 ml). The column, Waters Symmetry C18 (100 mm × 2.1 mm, particle diameter: 3.5 μm, Waters, Milford, MA, USA), and guard column, Security Guard C18 (4 mm × 2.0 mm, Phenomenex, Torrance, CA, USA) were kept in a column heater at 40 °C. The autosampler was kept at 4 °C, and the injection volume was 15 μl. Samples were analysed in a reversed sample order, with the sample incubated for 50 min analysed first, to minimise the effect of possible carryover effects.

A Micromass Quattro Premier triple quadrupole mass spectrometer (Waters, Milford, MA, USA) was used for detection. It was operated in positive ESI mode, with the following settings: capillary voltage, 3.0 kV; cone voltage, 35 V; extractor voltage, 5 V; source temperature, 110 °C; desolvation temperature, 400 °C; cone gas (nitrogen) flow, 100 l/h and desolvation gas (nitrogen) flow, 900 l/h. For MRM scans a resolution of 13, collision energy of 24 (WAY-100635), 22 (WAY-$d_5$ and MeO-WAY) and 26 (I-WAY) and a dwell time of 0.05 s were used. For quantification with MRM, the transition *m/z* 423.5 > 231.3 was used for WAY-100635, *m/z* 422.4 > 230.3 for WAB-$d_5$, *m/z* 447.4 > 255.3 for MeO-WAY and *m/z* 543.3 > 351.1 for I-WAY.

The concentrations of analogues and internal standard, obtained from in-house syntheses, were determined by selected ion recording with known concentrations of WAY-100635 for calibration, using the LC gradient described above. For LC–MS analysis in MS scan mode (*m/z* 50–500) of WAY-100635 obtained from a cold (non-labelling) chemistry synthesis and from a $^{11}$C-labelling batch an LC gradient was used as follows. At a flow-rate of 0.3 ml/min the initial setting of 5% B in A was changed to 40% B in 10 min. From 10 to 12 min the ratio of D was increased to 70%. The gradient was thereafter held at 70% D in 0.5 min, and returned to the initial setting from 12.5 to 13 min after the start of the program. This LC-program was also used in metabolite identification of WAY-100635 and MeO-WAY incubations.

Quantification in metabolic stability assays was performed using calibration samples consisting of 0.5, 1, 2, 3, 4 and 5 nM of WAY-100635 and the studied WAY-100635 analogue dissolved in a microsome solution, together with the internal standard at a concentration of 0.8 nM. MRM scans were used for quantification. Peak area ratios of analyte and internal standard were calculated and a weighted (1/*x*) linear regression curve was set up.

An investigation in the ability to quantify low concentrations of WAY-100635 was performed by analysing microsome calibration samples of concentrations 10, 20, 30 and 50 pM, with an internal standard concentration of 0.8 nM. Six injections of each concentration were carried out. A calibration curve was set up, and precision and accuracy were calculated as described above. The signal-to-noise ratio, S/N, was determined using MassLynx (ver. 4.0) software, calculating RMS values (the greatest height of the signal above the mean noise divided by the root mean square deviation from the mean of the noise).

### 2.4. Validation

Validation was performed by analysing microsome samples with known analyte concentrations on three different days. On each day quality control (QC) samples of concentrations 0.5, 2 and 5 nM WAY-100635 and MeO-WAY were prepared using commercially available WAY-100635 and MeO-WAY obtained from a cold (non-labelling) in-house synthesis. The internal standard, WAY-$d_5$, was added to a concentration of 0.8 nM. QC samples were analysed by six injections of each concentration and quantification was performed as described above. The correlation coefficient, $R^2$, obtained from the calibration curve was used to assess the linearity. The accuracy of the method was determined by calculating the ratio of the measured amount of analyte and the nominal value, multiplied by 100. The intra-assay precision was determined by calculating the R.S.D. of the six QC analyses of each concentration. Inter-assay precision was calculated as the R.S.D. of 18 QC analyses from three occasions.

## 3. Results and discussion

### 3.1. Mass spectrometry

For all the analytes (Fig. 1) pseudomolecular $[M + H]^+$ ions were detected, with *m/z* 422, 423, 447, and 543 for WAY-$d_5$,

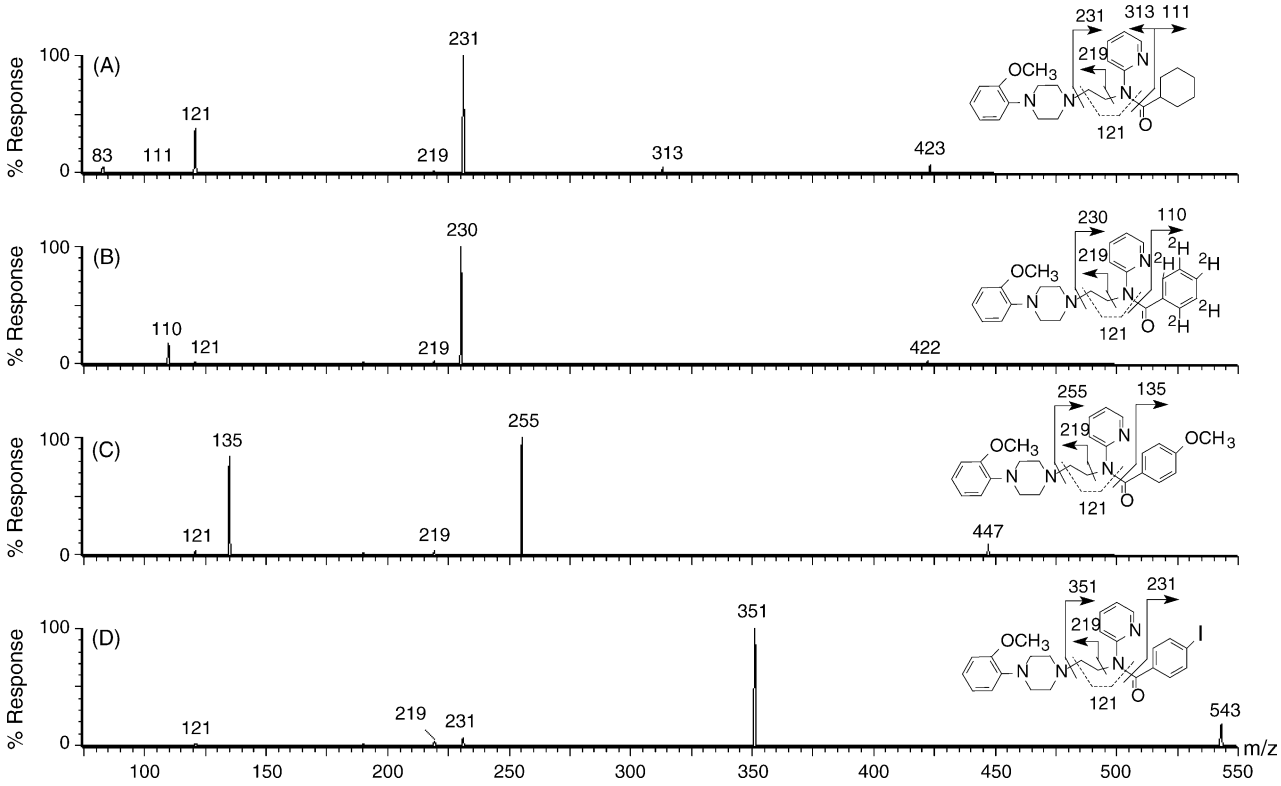



Fig. 2. Product ion scans of (A) WAY-100635 ($[M+H]^+$ *m/z* 423), (B) WAY-$d_5$ ($[M+H]^+$ *m/z* 422), (C) MeO-WAY ($[M+H]^+$ *m/z* 447) and (D) I-WAY ($[M+H]^+$ *m/z* 543).

WAY-100635, MeO-WAY and I-WAY, respectively. Product ion scans were recorded from the $[M+H]^+$ ion of each analyte and proposed fragmentation paths were outlined (Fig. 2). The main peak in all spectra is suggested to be the result of a neutral loss of *m/z* 192, corresponding to the methoxyphenylpiperazine moiety. The formed charge-carrying fragment of this cleavage was used for quantification in MRM mode. Fragments generated by cleavage of the amide bond were also detected. For WAY-100635, this breakage gave low abundant cyclohexanecarbonyl carbocations of *m/z* 111, but also, with hydrogen rearrangement, the ion corresponding to the other half of the molecule of *m/z* 313 (Fig. 2A). For the other analytes, the amide bond cleavage generated corresponding acyl carbocations of *m/z* 110, 135 and 231, but no other fragments could be detected. In the case of WAY-$d_5$, MeO-WAY and I-WAY, these acyl carbocations were stabilised by resonance effects. This was particularly pronounced for MeO-WAY, where the oxygen in the methoxy group provides additional electrons for stabilisation. The resulting peak at *m/z* 135 was almost as abundant as the *m/z* 255 peak (Fig. 2C). Cleavage of both the amide bond and the bond between the piperazine nitrogen and the ethyl unit was observed for all analytes, generating a fragment of *m/z* 121. Additionally, the peak at *m/z* 219, common to all analytes, is suggested to be the result of a disruption of the bond between the carbon in the ethyl unit and the amide nitrogen. For WAY-100635, the peak at *m/z* 83 most likely corresponded to a cyclohexane carbocation.

### 3.2. LC–MS

Due to the close proximity in mass-to-charge ratio between WAY-100635 and WAY-$d_5$ ($\Delta m/z = 1$), and to the similar fragmentation paths, a significant contribution from $^{13}$C-WAY-$d_5$ was seen in the MRM channel of WAY-100635. This can be seen in Fig. 3, where 10 pM WAY-100635 in microsome solution was analysed with 0.8 nM of WAY-$d_5$. A baseline separation

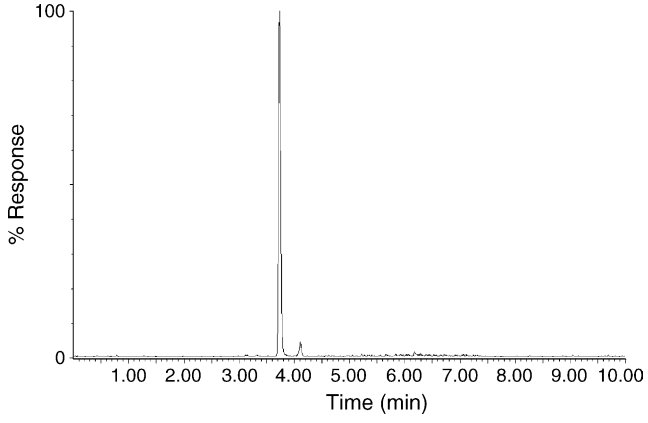



Fig. 3. LC–MS/MS chromatogram of MRM transition 423 > 231, used for quantification of WAY-100635. Analysis of a microsome sample of 10 pM WAY-100635 and 0.8 nM WAY-$d_5$. The first peak is a result of the contribution from $^{13}$C-WAY-$d_5$ and the second peak is WAY-100635.

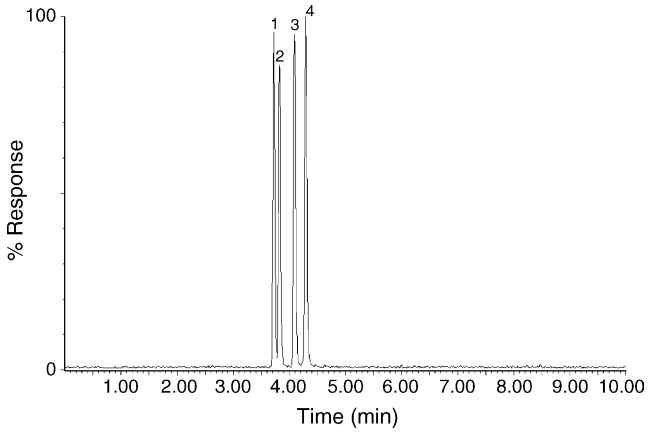



Fig. 4. LC–MS/MS (MRM) chromatogram of analysis of a microsome sample containing 1 nM WAY-100635, MeO-WAY and I-WAY. WAY-$d_5$ was added to a concentration of 0.8 nM. Acetonitrile was thereafter added to a concentration of 20%. Peak 1: WAY-$d_5$, 2: MeO-WAY, 3: WAY-100635 and peak 4: I-WAY.

was therefore necessary between WAY-100635 and the internal standard. As can be seen in Fig. 4, displaying a chromatogram of all analytes, this was clearly accomplished, thereby removing interfering signals of WAY-$d_5$ from the WAY-100635 channel. Additionally, blank microsome samples displayed no interfering matrix signals.

The carryover, determined by the injection of a 5 nM microsome sample followed by a microsome blank, was less than 0.2% for all analytes. Subsequent samples in the study were analysed in a reversed order, starting with low concentration samples, to reduce the signal contribution, however minimal, from the previously injected sample. Matrix effects were studied by comparing the response of the analyte dissolved in water and in a microsome solution. It was less than 10% for all analytes.

The sensitivity was investigated by using a calibration curve of lower concentrations (10–50 pM) than in the validation study. This enabled determination of 10 pM of WAY-100635 to a concentration of $10.2 \pm 0.96$ pM (±S.D.) (Fig. 3), which represented an accuracy of 102%. The R.S.D. was 9.4% and the average S/N was $27 \pm 5$.

### 3.3. Validation

The method was validated with respect to linearity, precision and accuracy in the concentration range 0.5–5 nM. These concentrations refer to actual concentrations in the incubation solutions. Subsequent addition of internal standard and acetonitrile led to a dilution with a factor of 0.75.

Good linearity was obtained for all analytes, with average $R^2$ values of $0.989 \pm 0.006$, $0.994 \pm 0.004$ and $0.996 \pm 0.003$ for WAY-100635 ($n = 3$), MeO-WAY ($n = 3$) and I-WAY ($n = 2$), respectively. The following average regression line equations were obtained: $1.44 \pm 0.19x - 0.15 \pm 0.06$, $1.03 \pm 0.07x - 0.10 \pm 0.03$ and $1.21 \pm 0.23x - 0.10 \pm 0.01$, for WAY-100635, MeO-WAY and I-WAY, respectively. The inter-assay R.S.D. of calibration standards was less than 7 and 6% for WAY-100635 and MeO-WAY, respectively, while the bias was at most 6% for WAY-100635 and 8% for MeO-WAY (Table 1). For I-WAY, where calibration curves were set up on two occasions, the maximum bias was 7%.

For QC samples, the intra-assay precision was determined to a R.S.D. value that did not exceed 6.4% for WAY-100635 and 4.1% for MeO-WAY (Table 2). The inter-assay precision was in the same range, with a maximum R.S.D. of 6.8 and 4.0% for WAY-100635 and MeO-WAY, respectively. The bias was at most 3% for WAY-100635 and 8% for MeO-WAY (Table 2).

The results demonstrate that high sensitivity, with good precision and accuracy, can be reached with the method. The amount of substrate obtained from $^{11}$C-labelling batches was 0.3 μg

Table 1
Inter-assay precision[a] and accuracy of calibration standards[b]

| Compound | Nominal concentration (nM) | Concentration found (nM) | R.S.D. (%) | Accuracy (%) |
|---|---|---|---|---|
| WAY-100635 | 0.5 | 0.52 ± 0.01 | 2.6 | 104 |
| | 1 | 0.99 ± 0.04 | 4.4 | 99 |
| | 2 | 1.96 ± 0.10 | 5.0 | 98 |
| | 3 | 3.0 ± 0.15 | 4.9 | 100 |
| | 4 | 3.72 ± 0.15 | 4.0 | 93 |
| | 5 | 5.31 ± 0.35 | 6.5 | 106 |
| MeO-WAY | 0.5 | 0.54 ± 0.02 | 4.2 | 108 |
| | 1 | 0.96 ± 0.06 | 5.8 | 96 |
| | 2 | 1.94 ± 0.03 | 1.5 | 97 |
| | 3 | 2.85 ± 0.09 | 3.3 | 95 |
| | 4 | 3.95 ± 0.12 | 2.9 | 99 |
| | 5 | 5.26 ± 0.17 | 3.2 | 105 |

[a] Given as R.S.D.
[b] Data is based on two repeated injections of each concentration, replicated on three different days.

Table 2
Intra- and inter-assay precision[a] and accuracy of quality control samples ($n = 18$[b])

| Compound | Nominal concentration (nM) | Concentration found (nM) | Accuracy (%) | Mean intra-assay precision (R.S.D.%) | Inter-assay precision (R.S.D.%) |
|---|---|---|---|---|---|
| WAY-100635 | 0.5 | 0.52 | 103 | 2.7 | 3.6 |
| | 2 | 1.93 | 97 | 3.9 | 5.8 |
| | 5 | 5.14 | 103 | 6.4 | 6.8 |
| MeO-WAY | 0.5 | 0.54 | 108 | 2.1 | 4.0 |
| | 2 | 1.91 | 96 | 2.5 | 3.0 |
| | 5 | 5.10 | 102 | 4.1 | 4.0 |

[a] Given as R.S.D.
[b] Data is based on six repeated injections of each concentration, replicated on three different days.

(0.6 nmol) for WAY-100635, 4 μg (10 nmol) for MeO-WAY and 7 μg (17 nmol) for I-WAY. With the high sensitivity obtained these amounts are sufficient for both validation and repeated metabolic stability tests.

### 3.4. Metabolic stability

In the initial method development an on-line extraction system was used for extraction of microsome solutions at a concentration of 1 mg/ml, as previously described [22]. The metabolism of WAY-100635 proved to be very rapid however, with less than 5% of the compound intact after 5 min of incubation. The protein concentration of the microsome solution was therefore lowered to 0.05 mg/ml to obtain a biological system with a slower and more measurable transformation rate. Direct injection, after addition of acetonitrile and centrifugation, of the sample onto the analytical column was thereafter possible, due to the low protein concentration. This afforded an analytical method of greater simplicity and was therefore preferred.

Having established a method with high precision and accuracy quantification in the lower nM range, it was investigated whether similar biological responses would be obtained with a metabolic stability assay using substrates from a $^{11}$C-labelling batch or a cold (non-labelling) chemistry synthesis. In Fig. 5, it can clearly be seen that the $^{11}$C-labelling batch of WAY-100635 contained a number of impurities that were not present in a cold chemistry obtained WAY-100635 solution (commercial source). Metabolic stability data for WAY-100635 and MeO-WAY are presented in Table 3. No significant differences could be seen in the metabolic stability between WAY-100635, obtained from a $^{11}$C-labelling batch source and a cold chemistry synthesis. Similar results were obtained for MeO-WAY (Table 3). These results indicate that ultra-pure substrates are not needed for the stability assay. This entails that an approach of fast $^{11}$C-labelling method development can be used, without extensive development time spent on purification procedures.

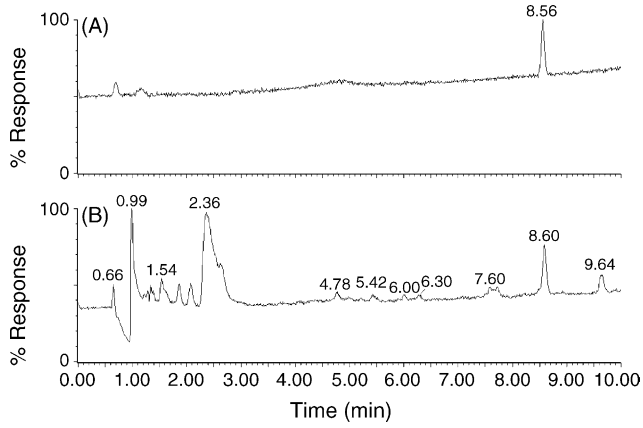



Fig. 5. Chromatogram of 0.7 μM WAY-100635 obtained from (A) a cold (non-labelling) chemistry synthesis (commercial source) and (B) a $^{11}$C-labelling batch (in-house synthesis). WAY-100635 was detected with a retention time of 8.6 min. MS scan *m/z* 50–500 in positive ESI mode.

WAY-100635 was co-incubated with the investigated analogue in all metabolic stability tests as a quality control, to ascertain that the microsomal enzymes were in good working condition. Apart from MeO-WAY, the stability of another analogue, I-WAY (Fig. 1), obtained from a $^{11}$C-labelling batch, was determined. In Fig. 6, data from repeated incubations with WAY-100635, MeO-WAY and I-WAY is plotted. It can clearly be seen that MeO-WAY displayed an increased resistance to metabolic transformation compared to WAY-100635. The amount of intact MeO-WAY that remained after 50 min of incubation was on average 2 times that of WAY-100635. I-WAY, on the other hand, displayed a decreased stability compared to WAY-100635. This can most likely be attributed to degradation by dehalogenation, a common metabolic pathway [32]. After 50 min of incubation the concentration of unchanged I-WAY was on average 50% of the concentration of WAY-100635. The results show that the method can be used for sensitive and efficient in vitro screening of metabolic stability of compounds directly obtained from a $^{11}$C-tracer protocol.

Table 3
Metabolic stability (relative amount of intact compound) of WAY-100635 and MeO-WAY obtained from cold (non-labelling) chemistry syntheses and from $^{11}$C-labelling syntheses

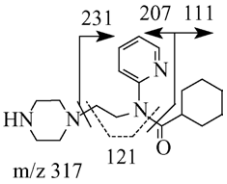
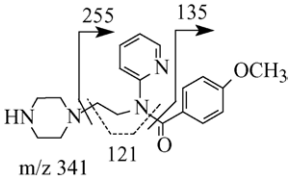
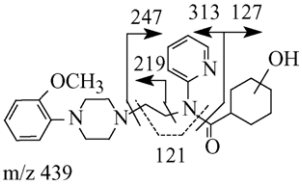
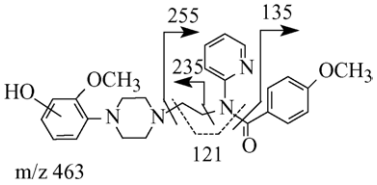
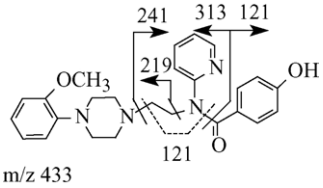
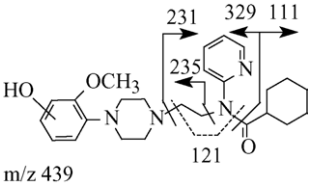
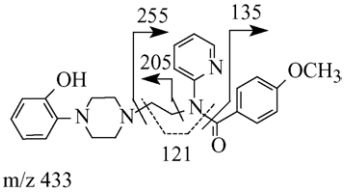
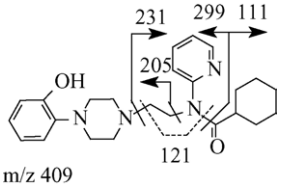
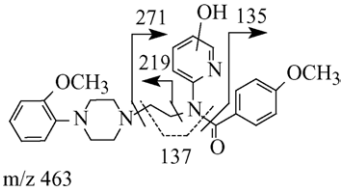
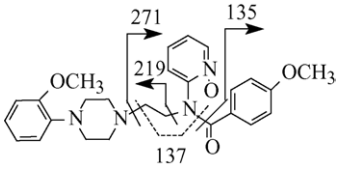
| Time (min) | Intact substrate (%) | | | | | |
|---|---|---|---|---|---|---|
| | WAY-100635 | | | MeO-WAY | | |
| | Cold[a], day 1 | Cold, day 2 | $^{11}$C[b] | Cold[a], day 1 | Cold[a], day 2 | $^{11}$C[b] |
| 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| 5 | 97 | 94 | 93 | 98 | 100 | 98 |
| 10 | 95 | 85 | 88 | 99 | 95 | 96 |
| 15 | 80 | 75 | 76 | 91 | 90 | 90 |
| 20 | 68 | 69 | 71 | 88 | 88 | 88 |
| 30 | 49 | 50 | 51 | 79 | 80 | 76 |
| 40 | 42 | 39 | 43 | 77 | 73 | 74 |
| 50 | 31 | 27 | 30 | 67 | 58 | 66 |

Experiments were performed on 2 days for cold chemistry substrates and on 1 day for $^{11}$C-labelling synthesis substrates, with co-incubation of WAY-100635 and MeO-WAY.

[a] Cold (non-labelling) chemistry synthesis.

[b] $^{11}$C-labelling synthesis.

Table 4
Detected ions in LC–MS analysis of 2 h microsome incubation of WAY-100635 and MeO-WAY

| WAY-100635 | | MeO-WAY | |
|---|---|---|---|
| LC Peak[a] | Precursor (metabolite) and product ion | LC Peak[b] | Precursor (metabolite) and product ion |
| I |  m/z 317 | I |  m/z 341 |
| II + III |  m/z 439 | II |  m/z 463 |
| | | |  m/z 433 |
| IV |  m/z 439 | III |  m/z 433 |
| V |  m/z 409 | IV |  m/z 463 |
| | | |  |

Product ion scans: *m/z* 100–450 (WAY) and 100–650 (MeO-WAY). Proposed structures of precursor (metabolite) and product ions are listed.
[a] See: Fig. 7A.
[b] See: Fig. 7B.

### 3.5. Metabolite identification

In order to probe the identity of the formed metabolites, rat liver microsome incubations of WAY-100635 and MeO-WAY were analysed with LC–MS in scan mode (Fig. 7). Precursor ions were selected from the resulting metabolic peaks and product ion scans were performed. In Table 4, detected precursor and product ions of WAY-100635 and MeO-WAY incubations are displayed with proposed structures. The suggested WAY-100635 metabolites were formed by dearylation by cleavage of the bond between the 2-methoxyphenyl and piperazine groups (Fig. 7, peak AI), hydroxylation of the cyclohexane ring (peak AII and AIII) and the methoxyphenyl ring (peak AIV), and demethylation (peak AV). Radiolabelled desmethyl-WAY-100635 has previously been detected in low amounts in plasma, after administration of [carbonyl-$^{11}$C]WAY-100635 to human volunteers [10].

For MeO-WAY (Table 4), a metabolite formed by the same dearylation pathway as for WAY-100635 was detected (Fig. 7, peak BI) and similarly, hydroxylation and demethylation in the 2-methoxyphenyl ring (peak BII and BIII). No hydroxylation in the *para*-methoxybenzamide group was however detected, but

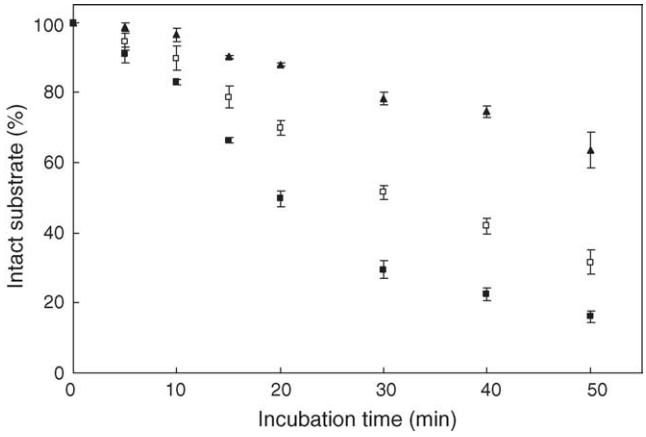



Fig. 6. Microsomal metabolic stability of MeO-WAY (▲), WAY-100635 (□), and I-WAY (■). Error bars represent one standard deviation, with the following number of experiments performed: WAY-100635, $n = 5$, and MeO-WAY, $n = 3$. For I-WAY, error bars represent max/min values, with $n = 2$.

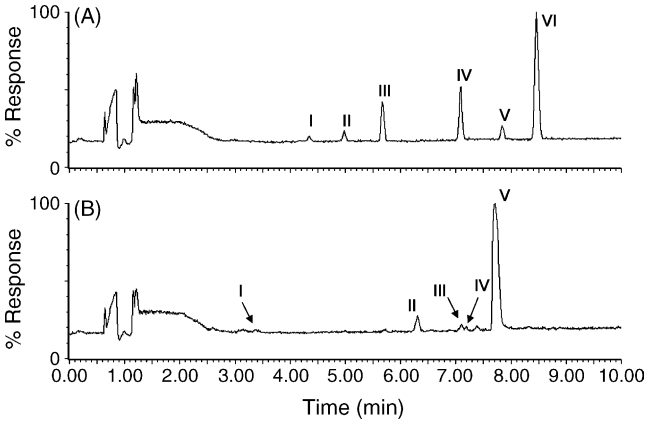



Fig. 7. LC–MS chromatograms obtained from analysis of 2 h rat liver microsome incubation with (A) WAY-100635 and (B) MeO-WAY. MS scan *m/z* 100–450 in (A) and *m/z* 100–650 in (B), positive ionisation. Peaks I–V in (A) represent metabolites and peak VI corresponds to WAY-100635. Peaks I–IV in (B) represent metabolites and peak V MeO-WAY.

instead demethylation in this group (peak BII). Additionally, oxygen was added to the pyridinyl ring, either by hydroxylation or N-oxidation, or by both (peak IV). Consistent with previous in vivo and in vitro rat metabolic studies of WAY-100635 [33] and fluoro-analogues [29,30], metabolites resulting from amide cleavage, the main pathway in human liver microsomes [13], were not detected. In this study, the applicability of the LC–MS method was demonstrated using liver microsomes obtained from rat. As a next step, human liver microsomes will be used to extend metabolic information regarding WAY-100635 and analogues.

## 4. Conclusions

The study demonstrated that the method can be used for sensitive metabolic stability analysis, of good precision and accuracy, of tracers obtained from a $^{11}C$ labelling synthesis batch. The results suggest that there is no need to perform additional cold (non-labelling) syntheses of compounds, but that these can be taken directly from a labelling experiment for determination of metabolic stability. Additionally, since the method uses detection of stable isotopes, experiments can be repeated using the material from a single synthesis. The result is a time efficient protocol for obtaining metabolic stability data of high quality.

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Financial support from the Swedish Research Council, contract no K5104-706/2001 (K M) and no K3464-345/2001 (B L), and Uppsala Imanet AB is acknowledged.

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