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Determination of flumazenil in human plasma by liquid chromatography–electrospray ionisation tandem mass spectrometry

M. Lavén[a], L. Appel[b], R. Moulder[b], N. Tyrefors[c], K. Markides[a,*], B. Långström[b,d]

[a] *Department of Analytical Chemistry, Institute of Chemistry, Uppsala University, Box 599, 75124 Uppsala, Sweden*
[b] *Uppsala Imanet AB, Box 967, 75109 Uppsala, Sweden*
[c] *Quintiles, Islandsgatan 2, 75318 Uppsala, Sweden*
[d] *Department of Organic Chemistry, Institute of Chemistry, Uppsala University, Box 599, 75124 Uppsala, Sweden*

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Abstract

A liquid chromatography–electrospray ionisation–tandem mass spectrometry (LC–ESI–MS/MS) method was developed to determine unlabelled flumazenil (Ro 15-1788) in human plasma in [^{11}C]flumazenil positron emission tomography (PET) studies. *N*-Methyl tri-deuterated flumazenil was used as an internal standard. The analyte and internal standard were extracted from plasma samples using solid-phase extraction, with a recovery of 78%. This was determined through the convenience of radioactivity measurements of ^{11}C-labelled flumazenil. The evaporated and reconstituted eluate was analysed by LC–ESI–MS/MS. The calibration curve was linear over the tested concentration range of 0.05–0.5 nM (15–150 pg/ml) with a correlation coefficient, R^2, of 0.998 ± 0.001. A high precision was achieved, with mean intra-assay and inter-assay relative standard deviations of at most 6 and 7%, respectively. The accuracy of the method ranged from 95 to 104%. As a proof of concept, the validated method was applied in the determination of flumazenil in plasma from two healthy volunteers participating in a PET study with three repeated investigations. A bolus-infusion protocol was used to achieve a constant concentration level of flumazenil. The average plasma concentrations ranged from 0.11 and 0.19 nM and all measurements were within the calibration standard range. The flumazenil concentrations were relatively constant within each scan and the average intra-scan precision was 15%.

Keywrod: Flumazenil

1. Introduction

Positron emission tomography (PET) is a sensitive tracer technique that utilises compounds labelled with short-lived positron emitting radionuclides, such as ^{11}C and ^{18}F, for obtaining reliable data of high precision for non-invasive in vivo studies. The technique is used in clinical diagnosis, but also as a means of studying drug interactions and receptor functions in connection with drug development [1–4]. Although PET provides a way to follow the course of radioactivity in a subject, it is not possible to differentiate between signals originating from intact labelled compound and from metabolites containing the radionuclide with positron emission detection. To overcome this problem methods based on the analysis of blood samples taken throughout a PET study have been developed to determine the proportion of intact tracer [5–7].

In our laboratory, the most common method to determine proportions of intact tracer is based on separation of metabolites and parent compound by liquid chromatography and detection by off-line radioactivity measurements. No absolute quantification is performed, but a relative measure of the amount of non-metabolised substrate is obtained, i.e. the ratio between intact substrate and total amount of radioactivity. This ratio can be utilised only if the metabolites have a totally different behaviour in vivo than the intact tracer. The advantages of this method lie in the high sensitivity and relative simplicity. However, a serious drawback is that the sensitivity rapidly decreases during the time course of the analysis and experiment due to the short half-life of the radionuclide (e.g. 20.3 min for ^{11}C). As a consequence, in a PET study precision and accuracy will be lower for data

* Corresponding author. Tel.: +46 18 471 3691; fax: +46 18 471 3692.
E-mail address: karin.markides@kemi.uu.se (K. Markides).

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obtained towards late time points. Moreover, the time restrictions allow analysis of only a limited number of samples with this quantification method. It is therefore of great interest to develop a method that is not bound to the short time limits that positron detection imposes on the analysis.

In the synthesis of a PET tracer only a fraction of the precursor is, in fact, labelled with the positron emitting radionuclide. This is due to isotopic dilution with the natural isotope which is present in the synthesis environment. If a highly sensitive detection technique is used, it should therefore be possible to quantify the unlabelled fraction of the radiolabelled tracer. Previously in our laboratory a packed capillary column liquid chromatography–electrospray ionisation–mass spectrometry (LC–ESI–MS) method was developed for the analysis of a PET tracer in human plasma [8]. This method was sufficiently sensitive for analysis in normal tracer dose range. By using LC–MS analysis of the stable isotope compound, measurements may be performed with a sensitivity which is, to the most part, constant with time. This could considerably increase the precision and accuracy of samples analysed at a late time point. In addition, since the short half-life time of the radionuclide is not a limiting factor a greater number of samples can be collected and each sample can be analysed in replicates or stored for later analysis.

[^{11}C]flumazenil ([^{11}C]Ro 15-1788) is a tracer used for quantification of benzodiazepine receptors in the human brain using PET [9–13]. A number of methods, based on radioactivity measurements, for the determination of the relative amount of non-metabolised [^{11}C]flumazenil in blood and plasma have been reported [7,12,14–16]. These suffer from the expected time restrictions imposed by ^{11}C analysis, which may result in poor accuracy and precision of the measurements. This can particularly affect the results of PET studies with [^{11}C]flumazenil where quantification is performed with mathematical compartment models using radioactivity measurements in plasma that are adjusted for the metabolism of the tracer [17].

Different methods have been published with respect to the quantification of unlabelled flumazenil in plasma. With gas chromatography–mass spectrometry the quantification of a plasma concentration of 0.5 ng/ml (2 nM) was reported [18]. Using liquid chromatography with ultraviolet detection approximately 2 ng/ml (7 nM) could be detected [19,20]. Whilst with a liquid chromatography–atmospheric pressure chemical ionisation–mass spectrometry method a limit of detection of 0.5 ng/ml (2 nM) was established [21]. In order to monitor the concentration of flumazenil typically encountered in plasma from PET studies, at least one additional order of magnitude of sensitivity is needed. To the authors' knowledge no method has been published where LC–ESI–MS is used for the determination of flumazenil in plasma.

The objective of the study was to develop an LC–ESI–MS/MS method for the determination of unlabelled flumazenil in the range of tracer concentrations in human plasma associated with PET studies with [^{11}C]flumazenil. For quantification of flumazenil in tracer doses the detection limit should be considerably lower than those of previously published methods. A method validation was performed with respect to linearity, precision and accuracy, using donated plasma from healthy volunteers. As a proof of concept, the validated method was applied in a PET study to determine flumazenil concentrations in plasma from two healthy volunteers.

2. Experimental

2.1. Materials

Solid-phase extraction discs SPEC PLUS^{TM} C18 AR (15 mg, 3 ml) were purchased from Ansys Technologies Inc. (Lake Forest, CA, USA). Formic acid (p.a.) was obtained from Merck (Darmstadt, Germany). Acetonitrile (Chromasolv) was obtained from Riedel de Haën (Seelze, Germany). Ammonium formate (AnalaR) was purchased from BDH Laboratory Supplies (Poole, UK). Ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a]-[1,4] benzodiazepine-3-carboxylate (flumazenil) was obtained from Hoffman-La Roche (Basle, Switzerland). *N*-Methyl tri-deuterated flumazenil (($^{2}H_3$)-flumazenil) and [^{11}C] flumazenil were synthesised in-house (Fig. 1) [22]. Plasma donated from healthy volunteers was obtained from Uppsala Blood Centre (Uppsala University Hospital, Sweden). In the PET study blood samples were withdrawn from two healthy volunteers during the PET investigations. The PET study was approved by the local ethics and radiation safety committees.

2.2. Sample preparation

The plasma (0.75 ml) was spiked at a level of 0.3 nM with ($^{2}H_3$)-flumazenil as an internal standard (Fig. 1). Sample preparation was handled by a Gilson ASPEC XL (Middleton, Wisconsin, USA) robotic system for solid-phase extraction (SPE). The extraction cartridge (SPEC PLUS^{TM} C18 AR) was conditioned with 0.5 ml acetonitrile followed by 0.5 ml 50 mM ammonium formate, pH 8.5. The sample was then loaded onto the cartridge, which was subsequently washed with 0.5 ml 95:5 (v/v) 50 mM ammonium formate,

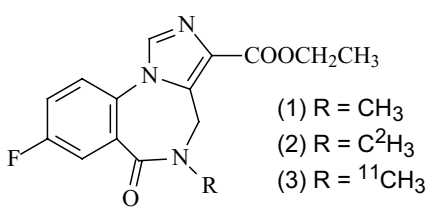

Fig. 1. Structural formulas of analytes. Compound (1): flumazenil (Ro 15-1788); compound (2): internal standard, ($^{2}H_3$)-flumazenil; and compound (3): [^{11}C]flumazenil.

pH 8.5/acetonitrile and eluted with 0.5 ml acetonitrile. The eluate was centrifuged for 1 min (20 000 × *g*), using an Eppendorf 5417R instrument (Eppendorf AG, Hamburg, Germany) operated at 4 °C, then transferred to an Eppendorf tube for approximately 1 h vacuum centrifugation (Labconco Centrivap Console, Kansas City, Missouri, USA). The dried sample was reconstituted in 100 μl 1:1 (v/v) 30% acetonitrile, 5 mM aqueous formic acid/water and transferred to a 700 μl glass autosampler vial. The sample was stored at −20 °C until LC–ESI–MS/MS analysis. Prior to sample injection the vial was centrifuged at 4400 × *g* for 3 min using a Heraeus Bifuge pico centrifuge (Heraeus, Hanau, Germany).

2.3. LC–ESI–MS/MS

A Shimadzu LC-10ADVP pump system (Shimadzu Corp., Tokyo, Japan) was used to deliver the two mobile phases consisting of 5 mM formic acid in water (A) and 5 mM formic acid in acetonitrile (B) at a flow rate of 200 μl/min. The pumps were programmed to generate a gradient running from 0 to 5 min with 10–70% of mobile phase B. The column, YMC Pro C18 (50 mm × 2.1 mm, Waters, Milford, MA, USA), and guard column, Securityguard C8 (4.0 mm × 2.0 mm, Phenomenex, CA, USA), were maintained at 40 °C within a column heater. Samples were injected employing a cooled (4 °C) Gilson 232 XL sample injector (Middleton, Wisconsin, USA) with an injection volume of 25 μl. The flow from the column was directed through a Rheodyne fluid valve which selected an outlet to waste or to a Micromass Ultima mass spectrometer (Waters, Milford, MA, USA) operated in the positive electrospray mode. Only the 2.2 min time window around the peak of interest was selected to the mass spectrometer in order to avoid unnecessary contamination of the interface. The following mass spectrometer settings were used: capillary voltage 3.2 kV, cone voltage 50 V, source temperature 80 °C, desolvation temperature 250 °C and collision energy potential 16 V. The scan mode used was multiple reaction monitoring, selecting precursor ions (M + H) 304.3 (flumazenil) and 307.3 (($^2H_3$)-flumazenil) and with the product ions *m*/*z* 257.8 and 260.8 used for quantification. The MS/MS fragmentation of flumazenil has previously been investigated employing an APCI ion source [23]. It was suggested that the path commenced with an initial loss of 28 Da from the ester chain followed by a loss of water producing an acylium ion (*m*/*z* 258).

2.4. Recovery of SPE step

The initial optimisation of the solid-phase extraction (SPE) step was performed with the aid of ^{11}C-labelled flumazenil (Fig. 1). The recovery was determined by radioactivity measurements in order to verify that no critical analyte losses occurred during sample clean-up and consequently that a sufficient amount of analyte was eluted in the final SPE step. To a blank plasma sample [^{11}C]flumazenil was added, resulting in a concentration of 0.4 nM. Subsequently ($^2H_3$)-flumazenil was added to obtain a concentration of 0.3 nM. This last step was performed so as to replicate PET investigation samples, where the addition of ($^2H_3$)-flumazenil could possibly lead to carrier effects. The recovery was established by measurements of the quantity of radioactivity in the sample before the start of extraction, the quantity in the eluate and retained on the column, as well as any quantities transferred to the wash liquid and the non-retained sample matrix. Radioactivity measurements were performed using a crystal scintillation counter [24], with a measure time of 60 s, and a limit of detection set to 15 counts per second. The procedure was performed on three occasions analysing a total number of 14 samples.

2.5. Validation

Validation was performed by analysing human plasma samples with known analyte concentrations on three different days. On each day 6 quality control (QC) samples of concentrations 0.10, 0.20 and 0.30 nM flumazenil were prepared and analysed together with calibration samples consisting of 0, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.40 and 0.50 nM flumazenil in plasma. The concentration range was chosen to reflect quantification of flumazenil in tracer doses associated with PET studies. In addition, two blank plasma samples were analysed on each occasion in order to detect possible interferents from the sample matrix. Quantification was performed by calculating peak area ratios of analyte and internal standard.

For each day calibration curves were constructed using non-weighted linear regression. The correlation coefficient, R^2, was used to assess the linearity of the calibration curve. The accuracy of the method was determined by calculating the ratio of the measured amount of analyte and the true value, multiplied by 100. The intra-assay precision was determined by calculating the relative standard deviation (R.S.D.) of the six QC samples of each concentration. Inter-assay precision was calculated as the R.S.D. of 18 QC samples from three occasions.

2.6. PET investigations

Two healthy male volunteers participated in the study with three separate PET investigations conducted during one day. The investigations were commenced in intervals of 3 h, with a duration of 60 min for each scan. Prior to the investigation an arterial catheter used for blood sampling was inserted in one arm under local anaesthesia. A bolus-infusion protocol was used to achieve a constant level of concentrations of labelled and unlabelled flumazenil in plasma. Therefore, the PET scan was started simultaneously with an intravenous bolus injection of 178 ± 9 MBq (±S.D.) [^{11}C]flumazenil and an intravenous infusion of 322 ± 28 MBq [^{11}C]flumazenil during 60 min. The specific radioactivity in the study was on average 52 MBq/nmol. Arterial blood samples were drawn

at 5, 10, 20, 40 and 60 min after start of the tracer injection. The plasma was separated from blood by centrifugation at 3100 × *g* during two min at 4 °C and was subsequently kept on ice before sample clean-up. The plasma samples, calibration standards and blank plasma samples were subsequently processed by SPE and stored in plastic Eppendorf tubes at −70 °C until LC–ESI–MS/MS analysis. Quantification, using peak areas of analyte and internal standard, was performed with non-weighted linear regression calibration curves. The results were evaluated on the basis of quantified plasma concentrations of flumazenil. The intra-scan precision of the measurements was assessed by calculating the R.S.D. of the five samples of each scan, assuming concentrations of flumazenil to be constant over time. The average intra-scan precision was determined as a measure of variability in precision between scans.

3. Results and discussion

3.1. Recovery of SPE step

The initial optimisation of the SPE step was performed conveniently with the aid of the radionuclide labelled analyte. In this way the recovery was established by measurements of the radiation from the different fractions of the clean-up procedure. In order to obtain accurate information from such measurements the radiochemical purity of the radiotracer must be kept high, since radiolabelled side products, with other chemical properties than the main compound, also contribute to the radiosignal. In the initial SPE optimisation a high radiochemical purity could be obtained, ranging from 96 to 98%, ensuring only minor side product radiosignals.

The recovery of the SPE step was thus determined as 78 ± 3.5% (±S.D.). The measurements revealed that analyte losses in the wash liquid and on the column were 5.4 ± 1.4 and 5.6 ± 3.4%, respectively. The quantity transferred to the non-retained sample matrix was negligible. An additional 10 ± 1.0% was unaccounted for, which likely corresponded to adsorption losses in the robotic liquid handling system. The recovery of the SPE step was considered to be sufficient to continue with the validation of the method.

3.2. Validation

The calibration curve was linear over the tested concentration range (0.05–0.5 nM), with an average correlation coefficient, R^2, of 0.998±0.001 (±S.D.), calculated from three calibration curves. The average slope and intercept were determined to 2.86 ± 0.07 and 0.065 ± 0.009, respectively. A high precision and accuracy were obtained in the analysis of calibration standards. The average inter-assay R.S.D. was 3.7 ± 4.9% and the accuracy was on average 101 ± 5.8% (Table 1). The inter-assay R.S.D. of the calibration standards was less than 6% and the bias was at most 3%, with the exception of the lowest calibration standard of 0.05 nM. This indicates that the 0.05 nM concentration is close to the limit of quantification. The US Food and Drug Administration state that the lowest standard on the calibration curve should be reproducible with a precision of 20% and an accuracy of 80–120% [25]. In this validation the lowest standard concentration could be determined within these limits.

For the QC samples the precision was generally very high (Tables 2 and 3). The highest intra-assay precision was obtained for 0.2 and 0.3 nM QC samples, yielding an average R.S.D. of 3% or less. Analysis of the lowest concentration QC samples (0.1 nM), resulted in a mean intra-assay R.S.D. of 6%, indicating a slightly lower precision. The inter-assay

Table 1
Inter-assay precision[a] and accuracy of calibration standards ($n = 3$[b])

Nominal concentration (nM)	Concentration found (±S.D.[c])	R.S.D. (%)	Accuracy (%)
0.051	0.059 ± 0.009	15	115
0.101	0.103 ± 0.001	0.7	102
0.149	0.151 ± 0.008	5.6	101
0.200	0.195 ± 0.005	2.6	97
0.253	0.258 ± 0.008	3.0	102
0.300	0.291 ± 0.003	1.1	97
0.400	0.390 ± 0.005	1.2	97
0.500	0.498 ± 0.001	0.2	100
Mean		3.7 ± 4.9	101 ± 5.8

[a] Given as the relative standard deviation (R.S.D.).
[b] Precision and accuracy data is based on three measurements for each concentration.
[c] Standard deviation.

Table 2
Intra-assay precision[a] and accuracy of quality control samples ($n = 6$[b])

Nominal concentration (nM)	Day	Concentration found (±S.D.[c])	R.S.D. (%)	Accuracy (%)
0.100	1	0.102 ± 0.009	8.3	102
0.100	2	0.095 ± 0.006	5.8	95
0.100	3	0.101 ± 0.004	3.7	101
Mean			6.0	
0.200	1	0.205 ± 0.008	3.8	102
0.200	2	0.208 ± 0.009	4.3	104
0.200	3	0.199 ± 0.002	0.9	99
Mean			3.0	
0.300	1	0.309 ± 0.008	2.7	103
0.300	2	0.305 ± 0.006	2.1	102
0.300	3	0.311 ± 0.012	3.9	104
Mean			2.9	

[a] Given as the relative standard deviation (R.S.D.).
[b] Precision and accuracy data is based on six measurements for each concentration, replicated on three different days.
[c] Standard deviation.

Table 3
Inter-assay precision[a] and accuracy of quality control samples ($n = 18$[b])

Nominal concentration (nM)	Concentration found (±S.D.[c])	R.S.D. (%)	Accuracy (%)
0.100	0.099 ± 0.007	6.8	99
0.200	0.204 ± 0.007	3.6	102
0.300	0.308 ± 0.009	3.0	103

[a] Given as the relative standard deviation (R.S.D.).
[b] Precision and accuracy data is based on 18 measurements of each concentration.
[c] Standard deviation.

precision, measured as the R.S.D., showed the same pattern and was 3–3.6% for the 0.2 and 0.3 nM samples and 6.8% for the 0.1 nM samples. A chromatogram of one 0.1 nM QC sample is shown in Fig. 2A. This chromatogram illustrates that the sensitivity of the developed method was high enough to determine 0.1 nM concentrations of flumazenil by LC–ESI–MS/MS.

The accuracy of the method ranged from 95 to 104% on the three different days for all concentrations of QC samples (Table 2). The average accuracy varied between 99 and 103%, implying a bias of at most 3% (Table 3). There was no relation between accuracy and concentration of the QC samples, indicating that relatively small systematic factors of the method had the same impact on the measurements for all QC sample concentrations.

The analysis of blank plasma samples displayed some interferents in the flumazenil m/z 304.3 > 257.8 transition (Fig. 2B). These could not be found in the solvent blanks, indicating that they emanated from the plasma. The multiple reaction monitoring detection mode was not sufficient to remove the signals of those endogenous compounds. Potentially, further development of the solid-phase extraction and liquid chromatography could result in the complete removal of the source of additional ions at this mass to charge ratio. However, considering the low intensity of these matrix signals, it was concluded that the separation was sufficient for this method.

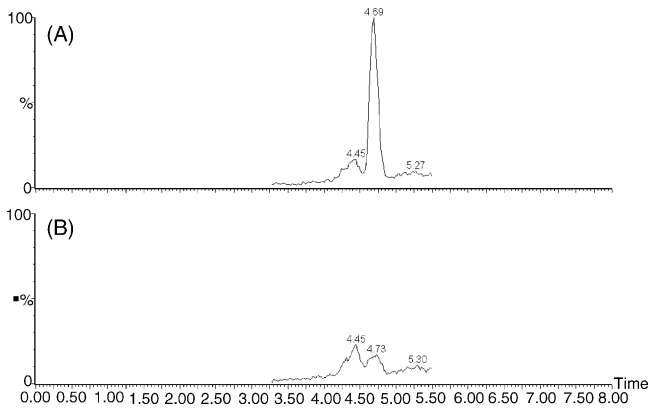

Fig. 2. Multiple reaction monitoring chromatograms of transition m/z 304.3 > 257.8. Chromatogram from (A) plasma sample spiked with 0.1 nM flumazenil; (B) blank plasma sample.

Table 4
Plasma concentration (nM) of flumazenil vs. time[a] in two healthy volunteers, each participating in three PET investigations

Time (min)	Subject 1			Subject 2		
	Scan 1	Scan 2	Scan 3	Scan 1	Scan 2	Scan 3
5	0.12	0.12	0.10	0.16	0.21	0.15
10	0.09	0.13	0.10	0.14	0.18	0.13
20	0.07	0.11	0.11	0.14	0.17	0.12
40	0.12	0.10	0.12	0.17	0.18	0.12
60	0.14	0.09	0.17	0.15	0.18	0.09
Mean	0.107	0.110	0.121	0.151	0.186	0.123
R.S.D.[b] (%)	27	14	23	9	8	19

[a] One sample was analysed per time point.
[b] Relative standard deviation.

For the internal standard, ($^2H_3$)-flumazenil, no interferents were detected in the m/z 307.3 > 260.8 transition. Furthermore, no carryover was detected, neither for flumazenil nor the internal standard.

3.3. PET investigations

The developed and validated method was successfully applied in the determination of flumazenil concentrations in plasma from two healthy volunteers participating in a PET study. The average plasma concentrations ranged from 0.11 and 0.19 nM for the six scans and all measurements were within the calibration standard range of 0.05–0.5 nM (Table 4). As expected the plasma concentrations of flumazenil were relatively constant within each scan. The intra-scan precision, measured as the R.S.D. of the five samples within each scan, varied between 9 and 27% and was on average 15 ± 8% (±S.D.). This is a good result with respect to the fact that in each investigation only five samples were taken and only one sample was analysed per time point.

Within scans the determined concentrations showed the smallest variation in the time frame between 10 and 40 min, due to reaching a steady state of flumazenil concentrations in plasma based on continuous infusion of [^{11}C]flumazenil during the PET investigation. One exception is scan 1 for subject 1 due to an erroneously low infusion rate during the initial 30 min. After correction of the flow rate the plasma concentration increased. For 5 and 60 min samples, in some cases the deviations from the average were somewhat larger than for the other time points, which might be the result of a non-steady state. Unexpectedly, the 60 min plasma sample in scan 3 of subject 1 had a flumazenil concentration that was considerably higher than previous samples of the same scan. The cause of this deviation could not be identified, but might illustrate a potential measurement error.

Between-scan variations of flumazenil plasma concentrations were not only caused by biochemical processes of the subjects and the analysis method, but also by the radiochemistry of [^{11}C]flumazenil and the tracer administration protocol. For each PET scan the specific radioactivity (amount of

radioactivity per mass) of [^{11}C]flumazenil and the injected amount of radioactivity were slightly different. As a consequence, somewhat different amounts of flumazenil were administered which resulted in average plasma concentrations that varied from scan to scan.

In this PET study the number of subjects included and samples per investigation was limited. Two healthy volunteers are generally not sufficient to estimate quantitative parameters in a PET study. On the other hand, both subjects had three PET investigations, resulting in totally six PET investigations. From a view of method development the number of volunteers and PET scans was considered to be sufficient to demonstrate that the method can be applied in vivo. For a quantitative analysis of PET data the number of volunteers should be extended. The other aspect was that due to few samples any sampling or measurement errors could have a relatively great impact on the intra-scan precision. However, the described LC–ESI–MS/MS method permits repeated sampling of each time point as well as increased sampling during the scan. Samples can be collected during a study and frozen for analysis at a later time point. Additionally, each sample can be re-analysed. This is generally not feasible with methods relying on radioactivity measurements due to the short half-life of ^{11}C. For future PET studies with [^{11}C]flumazenil we therefore intend to take full advantage of the developed method, using an increased number of samples, as well as repeated analysis of each sample, to increase the precision and accuracy of the measurements.

The results of this study showed that the developed LC–ESI–MS/MS method can be applied in a PET study to determine flumazenil concentrations in plasma with high precision and accuracy. As a next step, we intend to implement the described LC–ESI–MS/MS method and a conventional liquid chromatography radiodetection method in a larger scale [^{11}C]flumazenil PET study. This will permit a comparison of the two analytical methods by, for instance, assessment of the accuracy and precision of the ensuing quantitative PET data, such as the binding potential of [^{11}C]flumazenil to benzodiazepine receptors in different brain regions. The presented results indicate that the LC–ESI–MS/MS method could be a powerful complement to classical radiodetection methods and may contribute in improving the quality of quantitative results in [^{11}C]flumazenil PET studies.

4. Conclusions

In this study it was demonstrated that unlabelled flumazenil can be determined by LC–ESI–MS/MS in the lower nM tracer dose range in plasma and this concept was successfully applied in a [^{11}C]flumazenil PET study. Validation of the method showed a high precision and accuracy throughout a range of concentrations between 0.1 and 0.5 nM. In the PET study the measured plasma concentrations were all within the validated range of concentrations. The developed method is not restricted by the time limitations of ^{11}C-radionuclide measurements, which makes it possible to maintain a high sensitivity, and thus precision and accuracy, throughout the whole time course of a PET scan. The possibility to perform repeated and increased sampling could further increase precision and accuracy of measurements in vivo. The presented method could therefore be of great importance for the quality of quantitative results in [^{11}C]flumazenil PET studies.

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