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Determination of raclopride in human plasma by on-column focusing packed capillary liquid chromatography–electrospray ionisation mass spectrometry

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Abstract

A packed capillary liquid chromatography–electrospray ionisation mass spectrometry method was developed for the quantitative determination of raclopride in human plasma samples. Plasma samples containing the drug and its isotopically substituted analogue ($^{2}H_{3}$)raclopride as internal standard were extracted on solid-phase extraction discs, evaporated and reconstituted in a solvent with less elution strength than the mobile phase. Packed capillary columns of 100 mm×500 μm I.D. were used to obtain high mass sensitivity in the analysis and large volume injections (20 μl) were performed with analyte enrichment on top of the column. The assay exhibited satisfactory accuracy and precision over the concentration range of 0.2–15 n*M* (70–5200 pg/ml) with a limit of quantification of 0.2 n*M*. Raclopride in plasma was determined after intravenous injection in a positron emission tomography study performed in the tracer dose range.

Keywords: Packed capillary column; Mass spectrometry; Raclopride

1. Introduction

Positron emission tomography (PET) [1] is a non-invasive tracer technique, which can be used for in vivo studies of compounds labelled with short-lived β^{+}-emitting radionuclides. The radionuclides of major interest in PET are ^{15}O, ^{13}N, ^{11}C, ^{18}F and ^{76}Br with physical half-lives of 2.1, 10.0, 20.3, 110 min and 16 h, respectively. If the compound of interest is labelled with one of the above given isotopes, its regional uptake and pharmacokinetics can be studied. The position of the radiotracer in humans at the time of decay is monitored by an array of external radiodetectors surrounding the subject. With the use of adequate radiolabelled tracers, PET may be used to visualise a large number of different events in the body, such as blood-flow [2], energy metabolism [2] and neurotransmission [3].

The most serious drawback with PET, however, is that the radiolabelled analyte cannot be distinguished from its in vivo formed β^{+}-emitting metabolites. This is particularly troublesome for radiotracers with rapid metabolism. In order to determine the metabolic rate during the time course of the study, simultaneous assays of the radiotracer in different body fluids may be performed. This has been accom-

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plished by use of different chromatographic techniques [4,5]. One approach is to spike the sample with the co-eluting unlabelled tracer compound followed by injection onto a preparative liquid chromatography (LC) system. The spiking must be performed to obtain high enough concentrations for detection with UV absorption. When the radiotracer is separated from its labelled metabolites, the radioactive content of the analyte fraction is determined in a well counter. The main advantage as well as the main limitation with this analytical method is that it relies on radioactivity from rapidly decaying samples for the detection. The advantage is that detection can be performed with high sensitivity, as a large fraction of the total number of decays can be monitored within short time intervals. The disadvantage is that, in work with ^{11}C-labelled substances ($t_{1/2}$=20.3 min), the measurements can only be performed during a very limited time before the signal-to-noise level becomes too low for reliable analysis due to decay of radioactivity. The time available for analysis and possibly also the accuracy of the last determinations in the metabolic decay curve could be increased if some other detection technique could be used for the analysis.

Due to isotopic dilution from the ^{12}C analogue present during nuclide production and synthesis, the radiotracer product always contains a mixture of labelled and unlabelled compound. The ratio between them is determined as the specific radioactivity in Bq/mol. It should thus be possible to determine the unlabelled fraction of the tracer substance by other detection techniques than radiodetection, providing the tracer is not endogenous and that the required sensitivity can be reached.

Liquid chromatography coupled to mass spectrometry (LC–MS) is usually referred to as a sensitive detection technique for biologically interesting substances [6]. When electrospray ionisation (ESI) is used, this detection technique behaves to some degree as a concentration sensitive device [7]. Increased mass sensitivity will therefore be obtained if columns with small inner diameters are used. This is important to consider in work with limited sample amount, such as plasma samples.

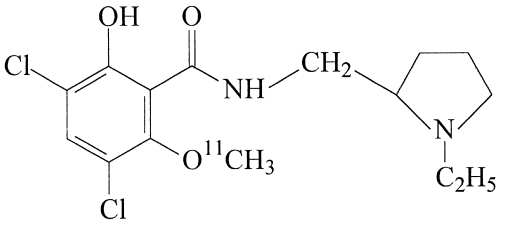
The aim of this study was to investigate if enough selectivity and sensitivity could be reached by packed capillary column LC–MS for the determination of 3,5-dichloro-6-hydroxy-*N*-[(1-ethyl-2-pyrrolidinyl)methyl]-2-methoxibenzamide (raclopride) in human plasma samples obtained from a PET investigation. Raclopride is a dopamine D_2 antagonist, which has been labelled with ^{11}C for PET studies of central dopamine receptor occupancy in healthy volunteers and schizophrenic patients [8]. The average value of specific radioactivity for ^{11}C-labelled raclopride after synthesis was determined to 95 GBq/μmol (n=50, values were in a range between 10 and 300 GBq/μmol). The average amount of radioactivity injected in these studies were 340 MBq, which corresponds to an initial plasma concentration of raclopride of 10 n*M* upon injection 10 min after synthesis. The expected raclopride plasma concentrations between 0 and 60 min after intravenous (i.v.) injection, may be calculated from determinations of radioactivity content in blood together with rate of metabolism. Based on earlier studies [9], the expected concentrations for [^{11}C]raclopride were estimated to be in the range of 0.1–10 n*M*. The detection limit in LC–ESI-MS, for raclopride in a standard solution injected on a 4.6 mm I.D. column was previously determined to 7 n*M* for a 20-μl injection [10]. This detection limit had to be significantly improved to make the plasma analysis possible.

In order to improve the mass sensitivity of the analysis, capillary columns of 500 μm I.D. were used in combination with large-volume (20 μl) injections. In addition, the sample was evaporated and reconstituted in a smaller volume after extraction. An isotopically substituted internal standard ($^2H_3$)raclopride, was used throughout the study (Fig. 1).

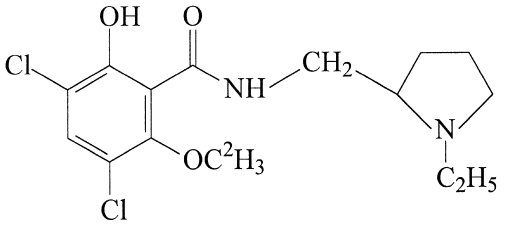
2. Experimental

2.1. Materials

Trifluoroacetic acid (TFA) and formic acid of analytical grade were purchased from E. Merck (Darmstadt, Germany). Acetonitrile and methanol of ultra gradient grade from Fisher Scientific (Loughborough, UK) and nanopure water (Elga Maxima, Bucks., UK) were used for mobile phases. Fused-



[*methoxy*-^{11}C]Raclopride



(*methoxy*-$^{2}H_3$)Raclopride

Fig. 1. Structural formulas of [methoxy-^{11}C]raclopride and (methoxy-$^{2}H_3$)raclopride.

silica tubing was obtained from Polymicro Technologies (Phoenix, AZ, USA). Deuterated methyl iodide (C^2H_3I), $^2H_3$ 99.5%, was purchased from Cambridge Isotope Labs. (Andover, MA, USA) and ammonium formate from Aldrich (Steinheim, Germany). 3,5-Dichloro-6-hydroxy-*N*-[(1-ethyl-2-pyrrolidinyl)methyl]-2-methoxibenzamide (raclopride) and 3,5-dichloro-2,6-dihydroxy-*N*-[(1-ethyl-2-pyrrolidinyl)methyl] benzamide (desmethyl-raclopride) were obtained from Astra Arcus (Södertälje, Sweden).

SPEC solid-phase extraction discs were purchased from Ansys (Irvine, CA, USA) and Dynagard 0.2-μm syringe filters from Spectrum Microgon (Laguna Hills, CA, USA). The packing material was 5 μm C_{18} particles from Kromasil (Phenomenex, CA, USA) and the column end frits were filters with 2 μm porosity from Vici (Schenkon, Switzerland).

2.2. Synthesis of internal standard

The synthesis of (methoxy-$^{2}H_3$)raclopride was performed in much the same way as the synthesis of [methoxy-^{11}C]labelled raclopride [9]. 3,5-Dichloro-2,6-hydroxy-*N*-[(1-ethyl-2-pyrrolidinyl)methyl] benzamide (15 mg, 45 μmol) and 21 μl 5 *M* NaOH were added to 500 μl of dimethylsulfoxide (DMSO) in a 2-ml reaction vessel. The vessel was heated at 140°C for 45 s followed by vigorous shaking until the solution turned dark green and 3 μl (6.5 mg, 45 μmol) methyl iodide (C^2H_3I) was added to the reaction mixture. The solution was then further heated at 85°C for 15 min.

Purification of the product was performed by two different preparative LC–MS methods on a 100×4.6 mm Kromasil C_{18} column. The first purification step was performed by isocratic elution with 20 m*M* TFA in water–acetonitrile (65:35, v/v) and the second step with 20 m*M* TFA in water–methanol (53:47, v/v). Identity of the purified product was then verified by injection together with unlabelled raclopride. The two compounds were shown to co-elute on a Kromasil C_{18} 100 mm×500 μm column in a buffer of 5 m*M* TFA in water–acetonitrile (70:30, v/v). Identity was also confirmed by LC–MS with electrospray ionisation where the protonated molecule $[M+H]^+$ of the product could be observed at *m*/*z* 350 as expected.

2.3. Sample preparation

Internal standard was added to all samples prior to extraction to give a sample concentration of 8 n*M*. The plasma samples, 1 ml, were then acidified by addition of 450 μl of 0.3 *M* formic acid. The samples were mixed by vortex and centrifuged for 5 min at 20 000 *g*. The supernatant was loaded on to a SPEC C_{18} solid-phase extraction disc, which had been pre-conditioned with 1 ml acetonitrile followed by 1 ml of 50 m*M* ammonium formate/formic acid (pH 3.5) in 3% (v/v) acetonitrile. Following the sample loading, the disk was washed with 1 ml of water–acetonitrile (90:10, v/v). The analytes were finally eluted with 1 ml of 20 m*M* TFA in water–acetonitrile (50:50, v/v). The eluting sample was passed through a 0.2-μm syringe filter attached to the SPEC tip before it was collected. The sample was then evaporated to dryness by freeze-drying (Centrivap Console; Labconco, Kansas City, MO, USA) and reconstituted in 100 μl of water–mobile phase diluted 50:50.

Human plasma samples were collected during a PET study between 6 and 60 min after i.v. injection.

Measurement of radioactivity was made in a NaI(Tl) well counter and the samples were then frozen at −70°C until they were used for further analysis. At the time of analysis the plasma was thawed and mixed with internal standard prior to extraction.

2.4. Packed capillary liquid chromatography

The mobile phase consisted of 10 m*M* TFA in water–acetonitrile (70:30, v/v). The analytical columns were packed with Kromasil 5 μm C_{18} material in 100 mm×500 μm I.D. fused-silica tubing. The packing material was suspended in 2-propanol and was allowed to stand in an ultrasonic bath for 10 min. The formed slurry was then pumped through the fused-silica tubing from a larger reservoir with a cone shaped outlet. Pumping was carried out using a Beckman 126 pump (Beckman Instruments, CA, USA) held at a constant pressure of 28 MPa for 2 h. Stainless steel filters with 2 μm porosity were used as column end frits.

A Beckman 126 pump was used for all analyses and was run at a flow-rate of 10 μl/min. The samples were injected in 20 μl volumes with a CMA autosampler (CMA/Microdialys, Stockholm, Sweden). A low dead volume transfer line was made from 30 cm fused-silica tubing (40 μm I.D.×110 μm O.D.), that connected the column with the electrospray probe. The fused-silica was inserted inside the electrospray steel capillary in order to reduce the dead volumes in the electrospray probe [11].

2.5. Mass spectrometry

The mass spectrometers used were a VG Platform and a VG Quattro (Micromass, Manchester, UK). Pneumatically-assisted electrospray ionisation was used in all experiments. Detection parameters in the mass spectrometer were determined by flow injection analysis of a solution of raclopride containing 10 m*M* TFA at a flow-rate of 20 μl/min. The capillary voltage was set to 3.0 kV and the cone voltage to 35 V. Detection was performed by selected ion monitoring of raclopride at m/z 347 and ($^2H_3$)raclopride at m/z 352.

2.6. Validation

The analytical method was validated by analysis of human serum quality control samples prepared at three different concentrations spanning the calibration range. Calibration samples together with six samples of each quality control pool were analysed on three different days. Precision and accuracy were determined. Intra-assay precision and inter-assay precision was determined from the variance obtained in analysis of variance (ANOVA) calculations and was expressed as the relative standard deviation (RSD) of each pool. Accuracy was measured according the following equation: percent difference from theoretical value=$[(X/C_T)-1]\cdot 100$, where X is the mean determined concentration of a quality control pool and C_T is the theoretical concentration.

2.7. Quantification

Each calibration curve consisted of 5–7 calibration points 0.2–15 n*M* (70–5200 pg/ml). Internal standard was added to all samples to give a concentration of 8 n*M*. Calculations were performed in the VG MassLynx NT 2.2 software (Micromass) on peak area ratios between the analyte and the internal standard. The calibration curve was calculated with linear regression without weights and the point of origin excluded. The limit of quantification was determined by running nine samples of a low concentration in series on the same day.

3. Results and discussion

3.1. Sample preparation

Elution of samples from SPEC discs generally requires low elution volumes as compared to other solid-phase extraction cartridges. A direct elution of the sample in 100 μl was, however, not possible for this particular analyte due to the lower recovery.

In order to remove any remaining proteins from the final sample, it was initially centrifuged at 20 000 *g* prior to injection. A pressure build-up in the separation column could, however, be seen during continuous sample injections. Every sample, therefore, had to be eluted from the extraction disc

through a 0.2-μm filter prior to evaporation. After filtration, there was less change in the column head pressure between injections and an increased number of samples could be injected on each column.

The absolute extraction recovery of raclopride was evaluated. Raclopride areas determined for two of each of the medium (2.5 n*M*) and high (10 n*M*) quality control samples were compared against the equivalent reference standards. Reference standards were prepared from raclopride dissolved in the same solvent as the extracted samples and injected directly on the separation column. The absolute recovery was calculated to be >95% (n=4).

3.2. Packed capillary liquid chromatography

As the ESI-MS response can be affected by differences in buffer composition between runs as well as co-eluting sample components [12], it is generally important to use chromatographic separation prior to detection in order to improve the reliability of the study [13]. The achievable detection limits when LC is coupled to a concentration sensitive detection technique, like ESI-MS, may be improved by injection of more sample on the chosen separation column or injection of the same sample volume on a column with reduced dimensions. In this study, the work was performed with limited human plasma sample amounts and a reduction of the column dimensions was therefore performed, by use of packed capillary columns of 500 μm I.D., while maintaining the injection volume (20 μl). Such a large injection is beneficial only if sufficient retention, and thus enrichment, of the analytes can be achieved on column. This was possible to obtain for raclopride when the final sample was dissolved in 5 m*M* TFA in 15% (v/v) acetonitrile followed by elution in 10 m*M* TFA in 30% (v/v) acetonitrile.

Packed capillary columns with an inner diameter of 200 μm and a flow-rate of 1 μl/min were initially used for the analysis. As the LC pumps could not deliver a stable flow-rate at 1 μl/min, the flow-rate was set to 50–200 μl/min and reduced in a linear split prior to the injection loop, directing 1 μl/min to the column. This set-up worked well as long as the back-pressure from the two split lines was constant. Upon repeated sample injections, however, the back-pressure from the column changed and therefore affected the split ratio. By use of this pre-injection split, it was thus impossible to maintain reproducible retention times. Columns with 500 μm I.D. were therefore used in the study, as the required flow-rate of 10 μl/min could be delivered from the pump without flow-splitting, making the separation method less sensitive to changes in back-pressure.

3.3. Mass spectrometry

To obtain the best sensitivity in detection with ESI-MS, it is desirable to work with low concentrations of mobile phase additives, while a too low concentration would result in insufficient control of the pH in the separation and thus a less robust method. In addition, raclopride shows pronounced peak tailing on silica-based reversed-phase columns. The ion-pairing effect and/or the reduced pH of a buffer containing 10 m*M* TFA was required to produce acceptable peak shapes for this study. It has been reported that TFA may cause signal suppression for certain analytes in electrospray ionisation [14,15], but for raclopride it has been shown that the sensitivity is roughly the same when TFA is exchanged for formic acid, while TFA reduces the peak tailing more efficiently [10].

The protonated molecules $[M+H]^+$ of raclopride and ($^2H_3$)raclopride could be detected at m/z 347 and m/z 350, respectively in positive electrospray ionisation. Due to overlap of natural isotope distributions between the two compounds, however ($^2H_3$)raclopride was determined at its second largest isotopic peak at m/z 352 (Fig. 2) in the entire study. The sensitivity of LC–MS as compared to LC–MS–

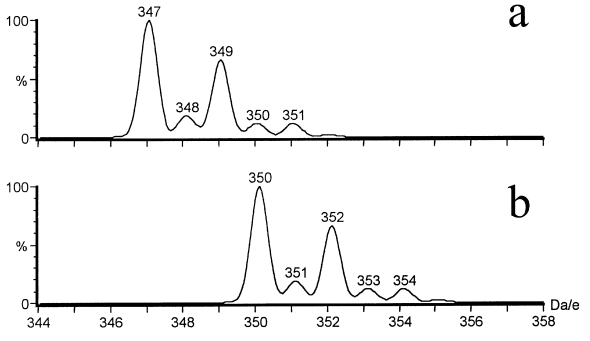


Fig. 2. Isotopic distribution of (a) raclopride and (b) (methoxy-$^2H_3$)raclopride.

MS was evaluated before the study was performed. It was shown that the sensitivity was six-times higher in LC–MS for this particular analyte. Although the increased specificity obtained in LC–MS–MS was valuable, the sensitivity would not have been enough for the current study and therefore LC–MS was used for all analyses.

3.4. Stability of the chromatographic system

The packed capillary columns showed slowly reduced separation efficiency upon repeated sample injections from the plasma extracts and was therefore exchanged after 50–60 injections. The sensitivity in the mass spectrometer proved to be unaffected only through some 30 samples. After this time the sensitivity was slowly reduced. As the quantification was performed with an isotopically substituted internal standard though, the reduction in sensitivity did not influence the quantitative results. The interface was cleaned between every sample series and contamination from non-volatile matrix material was then clearly visible on the high-voltage lens and the sampling cone. Increased stability of the columns as well as in the MS response may be obtained by a more selective sample preparation method.

3.5. Validation and quantification

The intra-assay precision of the study was 6–9%, the inter-assay precision was 16–24% and the mean accuracy was 3.5–3.7% as determined for each quality control pool of 18 samples (Table 1). The concentration range was chosen to reflect the concentration obtained in clinical studies after i.v. injection of raclopride.

A linear relationship between peak-area ratio of raclopride and ($^{2}H_{3}$)raclopride versus plasma concentration of raclopride was found in the investigated range of 0.2–15 n*M* at seven calibration levels. The limit of quantification was set to 0.2 n*M* (70 pg/ml), where the RSD was below 15%. The limit of quantification may be reduced by injection of even larger volumes or by the use of columns with further reduced dimensions.

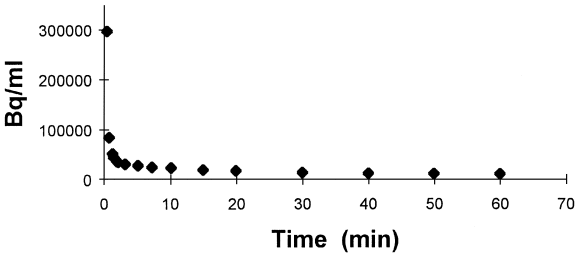


Fig. 3. Total radioactivity in plasma as a function of time, after an intravenous injection of [methoxy-^{11}C]raclopride.

Human plasma was collected during the course of a PET study for determination of raclopride metabolism. Total amount of radioactivity in blood was determined during 1 h after i.v. injection as shown in Fig. 3. Plasma samples were then analysed by the developed LC–MS method after total decay of the radiolabelled [^{11}C]raclopride. The sensitivity of the developed method proved to be high enough to determine the remaining amount of unlabelled raclopride by MS. Analysis of one such plasma sample, drawn 34 min after raclopride injection is shown in Fig. 4. The change in raclopride concentration with time is a function of the total plasma radioactivity and the metabolic rate. It could be seen that the rate of metabolism for raclopride during the time of investigation was slow in accordance with previously obtained results (Fig. 5) [9,16,17].

This study was performed to demonstrate the possibility to use MS rather than radiodetection for determination of raclopride in plasma during a PET

Table 1
Intra- and inter-assay precision and accuracy

Concentration (n*M*)	Mean accuracy (%)	Intra-assay precision (%)	Inter-assay precision (%)	Number of samples
0.56	3.5	7	16	18
2.51	3.7	9	24	18
10.1	3.5	6	19	18

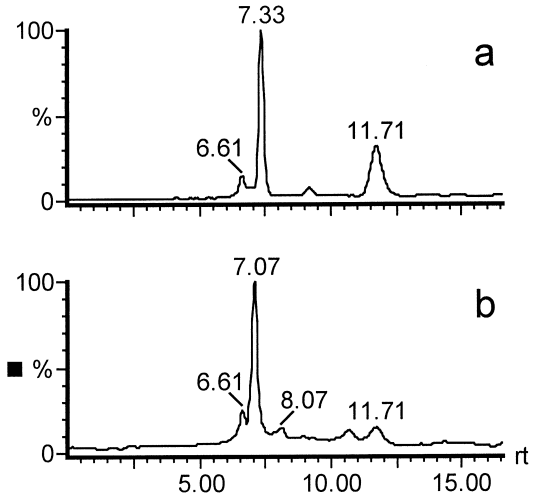


Fig. 4. Packed capillary LC–MS chromatogram of (a) the internal standard (methoxy-$^{2}H_{3}$)raclopride and (b) 0.25 n*M* raclopride in a plasma sample drawn from a healthy volunteer during a PET study. Raclopride eluted with a retention time of 11.7 min.

investigation. It could be shown that the use of MS provided several advantages. An increased number of samples could be analysed, during the same study. The stability in analysis was also more even for all investigated samples, as compared to radiodetection where the sensitivity and stability was quickly reduced with time due to the rapid decay of the radiotracer. And finally, samples collected during a study can be frozen and analysed at a later point in time, provided that the analyte stability under storage conditions are carefully investigated.

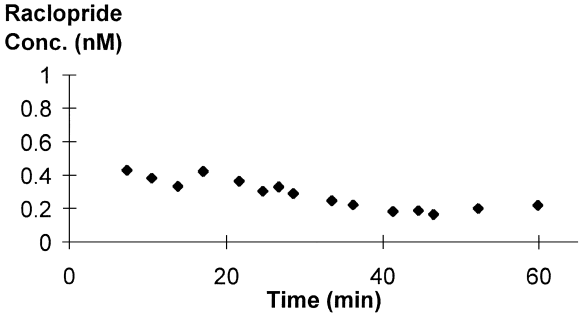


Fig. 5. Rate of raclopride metabolism was determined by packed capillary LC–MS in plasma samples drawn 6–60 min after i.v. injection.

4. Conclusion

The sensitivity and selectivity that could be reached with packed capillary column LC–MS enabled quantification of raclopride in human plasma samples withdrawn from patients participating in PET studies. This type of analysis has previously only been possible to perform by determination of radioactivity, which is a very sensitive detection method but is restricted by the 20.3 min half-life of ^{11}C. The possibility to quantify radiotracers from PET investigations with MS, had the distinct advantage that the time of radioactive decay was not limiting in the analysis.

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