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A Fast and Convenient Method for Robotic Preparation of [^{11}C]Flumazenil Avoiding HPLC Purification

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Summary

A new approach for robotic preparation of [^{11}C]flumazenil with recovery of the final product by SPE technique has been developed. A hydrogen-bond-assisted methylation in a suspension of potassium fluoride absorbed on alumina was followed by purification using a standard Sep-Pak Neutral Alum N Light cartridge. As a result, the contamination of the final product with its precursor Ro 15-5528 was reduced to similar levels as obtained by traditional semi-preparative HPLC. The synthesis of [^{11}C]flumazenil was operated by the commercially available robot Anatech RB-86. The formulated solution of [^{11}C]flumazenil was obtained within 18 min starting from trapping of [^{11}C]methyl iodide. The simplicity of this method and the use of disposable materials for purification instead of HPLC may be advantageous for routine clinical use.

Key Words: [^{11}C]flumazenil, automation, robotic synthesis, SPE purification, PET, carbon-11, central benzodiazepine receptors

Introduction

The availability of simple, fast and reliable production methods for the most commonly used radiopharmaceuticals (RPs) is a major prerequisite for the success of routine clinical application of PET. [^{11}C]Flumazenil (Ro 15-1788), a BZ receptor antagonist, is a well established radioligand for the in vivo quantitation of central benzodiazepine (BZ) receptors by PET [1-4]. In most of the reported methods the N-methylation of the desmethyl compound (Ro 15-5528) is carried out using [^{11}C]methyl iodide [5] or [^{11}C]methyl triflate [6] in acetone containing sodium hydroxide as a base.

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The isolation of pure [^{11}C]flumazenil from reaction mixture has been achieved by means of semi-preparative HPLC which provides an efficient separation of flumazenil from its desmethylated analogue. The affinity of Ro 15-5528 for BZ receptors is about 54 nM [7] and its presence in the formulated [^{11}C]flumazenil solution will result in a lower apparent specific radioactivity and may decrease the binding of [^{11}C]flumazenil to central BZ receptors.

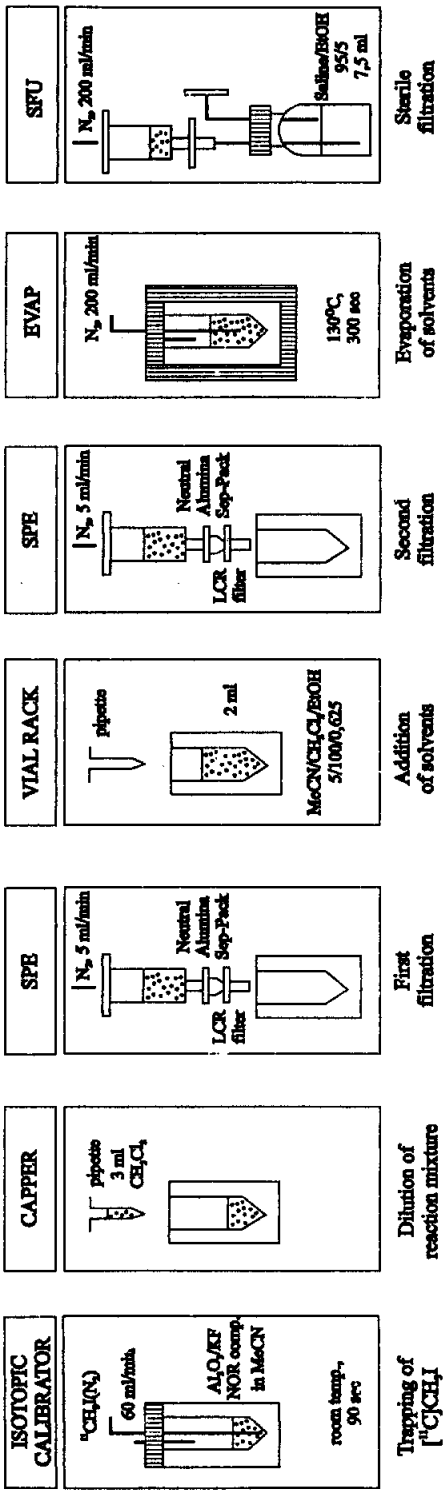
However, in routine production of RPs the use of semi-preparative HPLC is associated with longer synthesis time and lowered specific radioactivity, increased level of automation, requirement for additional space in hot cells and increased work-load to the personnel that handle the production system. In recent years the solid phase extraction (SPE) technique has, in combination with commercially available cartridges and various automated and semi-automated systems, found widespread applications in the preparation of PET RPs. A number of PET centers have recently been equipped with the commercially available Anatech RB-86 robotic system for the synthesis of both ^{18}F- and ^{11}C- labelled compounds. This system is provided with a set of work stations including a SPE unit compatible with commercially available cartridges [8]. The SPE purification step is performed under computer controlled conditions which provides the means for an efficient implementation of this separation technique as an alternative to HPLC purification. Robotic preparations of L-[methyl-^{11}C]methionine [9,10] and 1-[^{11}C]acetate [11,12] with recovery of the final product by SPE technique have recently been developed. The potential use of this methodology in the preparation of [^{11}C]flumazenil would present an important additional application for the efficient use of these robotic systems.

In the case of [^{11}C]flumazenil, the SPE purification step can be greatly facilitated when methylation is performed in a suspension of KF/Al_2O_3 in acetonitrile [13,14]. With this approach the major part of the precursor Ro 15-5528 is adsorbed on the surface of the solid support and can be easily separated from the product by simple filtration. The removal of residual amounts of precursor by the use of Neutral Alumina columns has been reported [15]. In-house preparation of Neutral Alumina columns as earlier described [15] is tedious, imprecise and may result in systems with variable flow characteristics. For routine preparations the application of commercially available cartridges is desirable. It is also considered as advantageous in modern practice for standardisation of methodology (ISO). The standard Sep Pak cartridges have been found to be useful in different steps of preparation of PET RPs. However, the separation of compounds with very similar properties, like desmethylated and methylated analogues, using these short cartridges is not a trivial task and has so far not been reported. The goal of this study was to find optimal conditions for the separation of flumazenil and its desmethyl precursor using the commercially available Sep-Pak Alum N Light cartridge and to develop a simple, fast and convenient method for robotic preparation of [^{11}C]flumazenil without HPLC purification.

Results and Discussion

The hydrogen-bond assisted methylation.

In recent years there has been a growing interest in utilising solid surfaces as reaction media for the preparation of various PET tracers. As was demonstrated by Yamawaki and Ando [16], *C*-, *N*-, *O*-, and *S*-alkylations can be conveniently performed in high yield under mild conditions by the use of potassium fluoride absorbed on various solid supports. This approach is well known as a "hydrogen-bond assisted alkylation". It has recently been applied to the synthesis of some carbon-11 labelled radioligands including flumazenil [13,14] and methionine [17]. In the presence of KF/Al_2O_3 as a solid support the methylation proceeds very rapidly, with high regioselectivity and radiochemical yield without formation of labelled by-products. In addition, this method makes it possible to remove the major part of desmethyl precursor when adsorbed on the surface of the solid support which greatly facilitates the further purification of the product. Both "on column" and "in suspension" methods have been applied for the synthesis of [^{11}C]flumazenil [13]. The methylation



Scheme 1. The main steps of robotic preparation of [^{11}C]flumazenil with the use of Anatech RB-86 (Method B).
SPE - solid phase extraction unit;
EVAP - evaporation station;
SFU - sterile filtration unit.

in a suspension of KF/Al_2O_3 in acetonitrile followed by filtration using a disposable filter was chosen in this study since it is easily adapted to robotic operation, provides a higher radiochemical yield and avoids in house-packing of the column with a solid support (Scheme 1).

After methylation and filtration of the suspension, the filtrate was analysed for the content of Ro 15-5528 by HPLC to determine the amount adsorbed on the solid support. The adsorption was measured in 15 experiments using commercially available KF/Al_2O_3 and the result, 94±8% (SEM), agrees well with the previously reported value, 97±5% (SEM), [14]. However, a high radiochemical yield (80-100%) in the methylation step was only achieved by using 1.1-1.3 μmoles of Ro 15-5528 instead of the reported amount of 0.75 μmole [13]. In our hands, the radiochemical yield dramatically decreased when the amount of precursor was less than 1 μmole. Drying of the solid support in an oven at 150°C for 24 hours as recommended [17] did not increase the yield in the methylation. As a result, the residual amount of precursor that had to be removed by SPE purification was about 20 μg instead of 0.7-1.6 μg as reported previously [13]. The discrepancy in the results may be attributed to the different amount of the surface water for different batches of commercially available KF/Al_2O_3. Although the role of the water remaining on the solid surface of the support in the methylation reaction is not completely understood, this parameter seems to be very important in the methylations performed on the solid surface as reaction media [16]. Interestingly, when acetone was used as a solvent instead of acetonitrile, the methylation yield dramatically decreased (down to 30-50%), and the portion of the precursor adsorbed on the solid support was only about 60%.

Optimization of the separation of flumazenil and Ro 15-5528 by SPE technique

In the case of N-[^{11}C]methyl amides, the separation of the N-methylated product from the precursor can be acheived with the use of small disposable Neutral Alumina columns (Scheme 1). In house dry-packed Neutral Alumina columns (3.2 mm x 180 mm bed) and solvents delivery system with miniature peristaltic pump have been used to remove relatively large amounts of the precursor [15]. For Ro 15-1788 the use of chloroform or its mixture with methylene chloride provided a good selectivity but the efficiency of separation was greatly influenced by a content of ethanol present in the chloroform as a preservative (about 1%) [15].

From several solvent systems investigated, we found that the mixture of $CHCl_3/CH_2Cl_2$ appeared to be a system of choice for the separation of flumazenil and Ro 15-1788 with the use of commercially available Sep-Pak Alum N Light cartridges. As was found in our preliminary study [18], the effectiveness of separation greatly depends on the ratio of the solvents and the filtration flow rate through the cartridge. With the use of Anatech RB-86 this parameter is computer controlled as the nitrogen flow rate applied to SPE station. The optimal separation was achieved when filtration flow through the cartridge was kept to a minimum (5 ml/min). The results of the model experiments with the use of a stock solution containing known amounts of flumazenil and Ro 15-5528 are shown in Table 1.

The results in Table 1 show that both flumazenil and Ro 15-5528 were completely trapped on the cartridge in pure methylene chloride or its mixture with chloroform, stabilized with 2-methyl-2-butene. The use of chloroform stabilized with 0.75% of ethanol in its mixture with methylene chloride (20/80 v/v) provided an efficient recovery of flumazenil from the cartridge while the major part of the precursor remained on the cartridge. Based on these data we assume that the presence of ethanol in this solvent system plays an important role in the process of separation. Interestingly, the mixture containing the same percentage of acetonitrile instead of chloroform with addition of a small amount of ethanol ($MeCN/CH_2Cl_2$ 20/80 v/v, 25 microliters of ethanol per 5 ml of mixture) gives a selectivity in the separation of Ro 15-5578 similar to that of $CHCl_3/CH_2Cl_2$. This finding is

of great practical importance since the same solvent (acetonitrile) may serve as a solvent in the alkylation and as a component of the solvent system for SPE purification. In this case evaporation of acetonitrile before SPE purification can be omitted which results in a shorter synthesis time. The optimal parameters obtained in these experiments (filtration flow-rate, solvent volume etc) were further used as variables in the ARC subprogram to operate the purification step of the radiosynthesis.

Table 1. Adsorption of Ro 15-5528 and flumazenil on Sep Pak Alum N Light cartridges in various solvent systems.

No	Solvent 1, %	Solvent 2, %	Ethanol, microliters per 5 ml	Ro 15-5528 trapped,%	Flumazenil trapped,%
1	$CHCl_3$ 100	CH_2Cl_2 0	0	0	0
2	$CHCl_3$ 0	CH_2Cl_2 100	0	100	100
3	$CHCl_3$ 40	CH_2Cl_2 60	0	25	13
4	$CHCl_3$ 20	CH_2Cl_2 80	0	90	30
5	*) $CHCl_3$ 20	CH_2Cl_2 80	0	100	100
6	$CHCl_3$ 20	CH_2Cl_2 80	25	92	8
7	MeCN 20	CH_2Cl_2 80	0	98	56
8	MeCN 20	CH_2Cl_2 80	25	97	15

*) in this experiment $CHCl_3$ stabilized with 2-methyl-2-butene was used;
in all other experiments $CHCl_3$ stabilized with 0.75% of ethanol was used.

The separation of [^{11}C]flumazenil from the desmethyl precursor by SPE technique

Two different systems - $CHCl_3/CH_2Cl_2$ (method A) and $MeCN/CH_2Cl_2/C_2H_5OH$ (method B) were used in the radiosynthesis of flumazenil. The first system with the earlier reported optimal ratio of the solvents in a mixture of 20/80 v/v [18] provided a good separation of [^{11}C]flumazenil from the precursor. Addition of small amounts of ethanol (25 μl in 5 ml of $CHCl_3/CH_2Cl_2$ mixture) facilitated the recovery of [^{11}C]flumazenil from the cartridge which reduced the losses of the labelled compound on the SepPak cartridge from 16-20 % [18] to 8-10 %. Using this method, the synthesis time was about 30 min starting from the trapping of [^{11}C]methyl iodide.

With the use of acetonitrile as a component of the solvent system for SPE purification, the evaporation step following methylation/filtration was omitted. In this case, filtration of the suspension through Millex FH and purification on Sep-Pak were combined in one step performed at the SPE station of RB 86 (Scheme 1). With this improvement the synthesis time was reduced to 18 min starting from the trapping of [^{11}C]methyl iodide. The best separation in the radiosynthesis was achieved with the following ratio of solvents: 4 ml CH_2Cl_2, 0.2 ml MeCN, and 25 µl of ethanol. The radioactivity distribution obtained in a typical preparation of [^{11}C]flumazenil by method B is shown in Table 2. The data presented are calculated at EOS.

Table 2. The radioactivity distribution obtained in a typical preparation of [^{11}C]flumazenil by method B (irradiation time 15 min with a beam intensity of 40 µA).

Activity of the product	140.8 mCi
Activity of the Sep-Pack	17.4 mCi
Activity of Millex FH	0.6 mCi
Activity of methylation vial	6.7 mCi
Activity of sterile filter	0.0 mCi

Both methods (A and B) give an amount of Ro 15-5528 in the range of 0.1 - 0.8 µg in 7.5 ml of total final product. This amount is similar to the levels obtained by semi-preparative HPLC [6]. The formulation of the product was obtained by the use of the EtOH/saline mixture (5:95 v/v). Radiochemical purity as measured by analytical radio HPLC was more than 99%. Specific radioactivity was in range of 0.3 – 1.6 Ci/µmol (11.1-59.2 GBq/µmol). The product did not contain any other UV detectable chemical impurities except the small amounts of the precursor. The product was obtained in a sterile and pyrogen free form and ready for injections. The content of residual solvents in the final preparation as measured by GC was less than 0.1 mg/ml for acetonitrile and less than 0.15 mg/ml for methylene chloride. These values are low when compared to the maximum daily dose recommended in the European Guidelines for residual solvent levels (4.1 mg for acetonitrile and 6.0 mg for methylene chloride per day [19]).

Experimental

Materials and General Methods

Materials: Potassium fluoride, 40 wt % on alumina and lithium aluminum hydride, 1.0 M solution in THF, were obtained from Aldrich; chloroform Baker Resi-Analyzed, stabilised by 0.75% of ethanol, and dichloromethane Baker Analyzed were purchased from Malinkrodt Baker Inc; chloroform Lichrosolv, stabilized with 2-methyl-2-butene was obtained from Merck. All solvents and chemicals were used without additional purification. Flumazenil, Ro 15-1788, and its precursor, Ro 15-5528, were kindly supplied by Dr.W.Hunkeler, La Roche, Basel, Switzerland. Sep-Pak Alum N Light (cat No 23561) cartridges were purchased from Waters-Millipore; Millex FH13 0.45 µm, Millex GV 0.22 µm, and SLGV R25LS filter units were obtained from Millipore. Supelco 6 mL filtration tubes (cat No 5-7242) were used in the SPE filtration unit.

Production of [^{11}C]CO_2, [^{11}C]CH_3I and methylation

Target irradiations were performed at the Institute of the Human Brain with a Scanditronix MC17 cyclotron producing 17 MeV protons. [^{11}C]CO_2 was formed via the $^{14}N(p,\alpha)^{11}C$ nuclear reaction in a target of nitrogen gas operated in a batch-wise mode with a beam intensity of 40 μA. [^{11}C]CO_2 produced was condensed in evacuated stainless steel coil immersed into liquid nitrogen and transferred by means of nitrogen flow through a P_2O_5 drying tube into the [^{11}C]CO_2/[^{11}C]CH_3I work station of the Anatech Robotic system. [^{11}C]Methyl iodide was produced by a standard method using $LiAlH_4$ and HI and was transferred to the reaction vessel in a stream of nitrogen gas at 30 ml/min through a short column filled by ascarite/sicapent (50/50 v/v) to remove the traces of HI and [^{11}C]methanol. [^{11}C]Methylations were performed in a 5 ml Weaton vial containing a suspension of 12±3 mg of KF/ Al_2O_3 and approx. 0.4 mg of Ro 15-5528 in 0.6 ml of acetonitrile. After trapping of [^{11}C]methyl iodide during 90 sec, reaction mixture was kept at room temperature for a further 120 sec.

Automation

All the synthetic operations were performed using the Anatech RB 86 robotic system (Anatech, Husbyborg, Uppsala, Sweden). The system is described in detail elsewhere [8]. It includes a lab robot with a grip hand, 1 ml and 5 ml syringes, evaporation block, solid phase extraction (SPE) and sterile filtration (SFU) units, wortex and capper stations, racks for 5 ml Weaton vials and 180 ml solvent bottles. The system was controlled by a Sequencer (programmable central processor SEQ 300) which in turn was controlled by a PC through the Anatech Robot Controller (ARC) software. The flow rate of carrier gas was controlled by a mass flow controller MFR 5850 (Brooks, Netherlands) operated by the ARC software. ARC programs were developed to operate the synthesis of [^{11}C]flumazenil and to optimise every step of the preparation.

Optimization of the separation of flumazenil and Ro 15-5528 by the SPE technique

A stock solution containing 32 μg of flumazenil and 10 μg of Ro 15-5528 in 0.5 ml of methanol was evaporated to dryness using a stream of nitrogen gas at 200 ml/min at 130 °C. The vial was cooled down, and its content dissolved in 2 ml of solvent. The solution was passed through a 6 ml Supelco tube equipped with a Sep-Pak Alum N Light cartridge previously conditioned by washing with 5 ml CH_2Cl_2. A second portion of solvent (2 ml) was added to a vial and then passed through the Supelco tube. The combined eluates were evaporated to dryness and the residue was dissolved in 8 ml of EtOH/saline (5/95 v/v). The procedure was controlled by the ARC subprogram used in the synthesis of labelled flumazenil. The final solution was analyzed to determine the content of flumazenil and Ro 15-5528.

Separation of [^{11}C]flumazenil from its precursor by the SPE technique

Method A [18]. The reaction mixture was passed through a 6 ml Supelco tube equipped with a Millex FH13 0.45 μm filter unit. After filtration, acetonitrile was removed using a stream of nitrogen gas at 200 ml/min during 150 sec at 130°C. The reaction vial was cooled down and its content was dissolved in 2 ml of $CHCl_3/CH_2Cl_2$ (20:80 v/v). The solution was passed through a 6 ml Supelco tube equipped with a Sep-Pak Alum N Light which had previously been washed with 5 ml of CH_2Cl_2. The filtration flow was kept to a minimum (applied nitrogen flow-rate was 5 ml/min). A

second two ml portion of $CHCl_3/CH_2Cl_2$ (20:80 v/v) was added to the reaction vial and the filtration step was repeated.

Method B. The reaction mixture was diluted with 3 ml of CH_2Cl_2 and directly passed through a 6 ml Supelco tube equipped with a Sep-Pak Alum N Light cartridge connected in series with a Millex FH13 0.45 μm filter unit. The Sep-Pak Alum N Light had previously been conditioned by washing with 5 ml of CH_2Cl_2. After filtration using conditions similar to method A, [^{11}C]flumazenil was eluted by passing a 2 ml portion of MeCN/CH_2Cl_2/EtOH (5/100/0.625 v/v/v) through the combination of Sep-Pak Alum N Light cartridge and Millex FH13 filter (see Fig.1).

For both methods SPE purification was followed by complete removal of the solvents in a stream of nitrogen gas at 200 ml/min during 300 sec at 130°C. [^{11}C]Flumazenil was dissolved in two portions of 4 ml of EtOH/saline (5/95 v/v) and the combined solutions were sterile filtered by passing it through a 0.22 μm Millex GV filter unit.

HPLC analysis of final product

The final product was analyzed for chemical and radiochemical purity with HPLC on a reversed phase Merck Lichrosphere 100 RP 18e column (4 mm x 250 mm, 5 μm,), using a Gilson Pump 305, a Rheodyne injector (20 μl loop), and a Gilson 116 UV absorbance detector in series with a Beckman 170 radiodetector. Peak analysis was done with a 4400 integrator (Varian, USA). The column was eluted with acetonitrile/0.01M phosphoric acid (25:75 v/v) at 1 ml/min and the eluate monitored at 254 nm. Under these conditions the retention times of Ro 15-5528 and flumazenil were 7.8 and 13 min respectively. The response of the UV detector was calibrated for mass by injection of known mass of authentic compounds.

GC analysis of final product

The final product was analyzed for the presence of residual solvents by GC on a Porapak Q column (3.2 mm x 1 m) using a gas chromatography system from Varian, USA. Helium was used as a carrier gas using a flow rate of 40 ml/min. Peak analysis was performed with a 4400 integrator (Varian, USA). The acetonitrile concentration was determined with a flame-ionization detector using the following conditions: column temperature 90°C; injector temperature 180°C; detector temperature 200°C. The retention time of acetonitrile and ethanol were 7.2 and 9.3 min respectively. The methylene chloride concentration was determined with a thermal conductivity detector using the following conditions: initial column temperature 90°C for 10 min; gradient 30°C/min for 3 min; injector temperature 180°C; detector temperature 200°C. The retention time of methylene chloride and ethanol were 6.6 and 10 min respectively. An external standard method was used to calculate the concentration of residual solvents in the analyzed samples.

Conclusion

The robotic synthesis of [^{11}C]flumazenil based on hydrogen-bond-assisted methylation followed by SPE purification provides a fast and convenient method for the preparation of this radiotracer. The method gives a final product with a Ro 15-5528 content in the range of 0.1 - 0.8 μg in 7.5 ml volume, and in high radiochemical purity and high specific radioactivity. The simplicity of this purification approach and the use of disposable materials are convenient and may be adapted to the non-robotic procedures previously developed [5,6].

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