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Analysis of L-[methyl-^{11}C]methionine and metabolites in human plasma by an automated solid-phase extraction and a high-performance liquid chromatographic procedure

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Abstract

A fully automated method for separation of L-[methyl-^{11}C]Methionine from metabolites in patient plasma was developed. L-[methyl-^{11}C]Methionine was isolated from plasma by solid-phase extraction (SPE). The radioactivity retained on the SPE column was eluted and injected onto the HPLC system for separation of in vivo formed L-[methyl-^{11}C]methionine radiolabeled metabolites. The yield through the isolation procedure and HPLC analysis was greater than 95% with a precision better than 5% (R.S.D.). The calculated rate of L-[methyl-^{11}C]methionine transport into tumor tissue was markedly different with and without compensation for radiolabeled metabolites in patient plasma.

Keywords: Methionine

1. Introduction

Positron emission tomography (PET) is an imaging diagnostic technique utilising tracers with short-lived radionuclides, such as ^{11}C ($t_{1/2}$=20.3 min), ^{15}O ($t_{1/2}$=2.03 min) and ^{18}F ($t_{1/2}$=110 min). The radionuclide is incorporated into a tracer by radiochemical synthesis. By appropriate choice of radiotracer the kinetics of a physiological or biochemical process in the living tissue can be measured in the normal or pathological state. Measurements of blood flow in the heart and brain [1], amino-acid transport into tumor tissue [2], receptor binding and neurotransmitter synthesis [3] are examples of processes commonly studied with PET. However, the radioactivity distribution measured with PET has to be processed to obtain quantitative information. The quantitation is based on compartment models which besides the tissue kinetics of radioactivity measured by the PET camera, also requires information of tracer concentration in a reference tissue, e.g., plasma.

The extent of metabolism of the tracer in tissue, such as the liver, depends on the chemical and biological properties of the tracer as well as the metabolic capacity of the patient. Measurement of total plasma radioactivity is inadequate for quantitation if the tracer is extensively metabolised or if the

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volume of distribution of the metabolites is small. Determination of the radioactivity emanating from the tracer vs. total plasma radioactivity is, therefore, essential for an accurate quantitation of the process. Fully automated bioanalytical methods used for separation of tracer and metabolites are required to ensure high capacity and reproducibility. Furthermore, the time required for complete analysis of the tracer in plasma has to be short due to the short physical half-life of the radionuclide.

L-[methyl-^{11}C]Methionine is one of several amino acids frequently used in oncology to study in vivo transport of the amino acid into the tumor tissue [4]. Quantitation of the process is calculated using a compartment model with the change of L-[methyl-^{11}C]methionine radioactivity in plasma over time as reference. The metabolism of L-methionine is complex [5,6] and several metabolites have been detected in plasma [7,8]. Large inter-individual variations have also been noticed regarding the amount of intact tracer in patient plasma during the time of the PET scan [7] and, therefore, individual correction for differences in L-[methyl-^{11}C]methionine plasma kinetics has been recommended [9]. Therefore, an automated method for clinical routine determination of L-[methyl-^{11}C]methionine vs. radiolabeled proteins and metabolites in plasma was developed.

2. Experimental

2.1. Chemicals and reagents

DL-Methionine and L-serine were purchased from BDH (Poole, UK) and Fluka (Buchs, Switzerland), respectively, and 4-methylthio-2-oxobutyrate and *S*-adenosyl-L-methionine were purchased from Sigma (St. Louis, MO, USA). NaCl (saline), trifluoroacetic acid (TFA), HCl and $HClO_4$ were obtained from Merck (Darmstadt, Germany). Ethanol was purchased from Kemetyl (Stockholm, Sweden) and NaOH from EKA Nobel (Surte, Sweden).

The radionuclide ^{11}C was produced in a Scanditronix MC17 cyclotron at Uppsala University PET centre and obtained as $^{11}CO_2$. The $^{11}CO_2$ was converted to $^{11}CH_3I$ and used in a reaction with *S*-benzyl-L-homocysteine [10]. Identity and radiochemical purity of L-[methyl-^{11}C]methionine was determined using HPLC. The final solution was passed through a 0.22-μm filter before intravenous administration of 150–800 MBq of L-[methyl-^{11}C]methionine to patients.

2.2. Apparatus

A laboratory bench robot device (ASPEC, Gilson, Middleton, WI, USA) was used for solid-phase extraction, injection of sample onto the HPLC system and finally fractionation of the eluent from the HPLC system for discontinuous measurement of radioactivity. A dilutor (Model 401, Gilson) was connected to the ASPEC and used as a dispenser in the sample isolation procedure. The computer controlled (Gilson 715) chromatographic system comprised a dual pump system (Model 306, Gilson) and a detector (Model 117, Gilson).

The radioactivity was measured for 60 s in a well-type scintillation counter with a NaI (Tl) crystal, an amplifier and a low energy discriminator.

2.3. Isolation of L-[methyl-^{11}C]methionine by solid-phase extraction

Empty disposable 3-ml SPE columns with 20 μm pore size frits in the bottom (Isolute Accessories, International Sorbent Technologic, Hengoed, UK) were packed with 500±10 mg cation resin AG50W-X4 (100-200 mesh, BioRad Laboratories, Richmond, CA, USA). The packed columns were centrifuged at 4000 *g* for 2 min (Beckman GS-15R centrifuge, Palo Alto, CA, USA), and thereafter each was conditioned with 1 ml of 70% ethanol and 0.1 *M* HCl. Plasma (1 ml) was applied onto the SPE column which was washed with 5 ml of deionized water (NANOpure, Barnstead, IA, USA). The radioactivity retained on the SPE column was eluted with 3 ml of 0.6 *M* NaOH into a tube containing 0.3 ml of 6 *M* HCl. The eluate was automatically mixed and injected onto the chromatographic system. The intermediate wash solution was measured for radioactivity.

2.4. High-performance liquid chromatography

Separation of methionine from metabolites was performed on a Bio-Scale S2 cation-exchange column (52×7 mm I.D., 10 μm particle size, BioRad

Laboratories) using 10 m*M* TFA with a pH of 2.1 as the mobile phase. The sample volume applied onto the column was 230 μl. A solution of 1 *M* saline was added to the mobile phase by a second pump starting at 5%, increasing to 70% and down to 5% (Fig. 1). The flow-rate of the mobile phase was 1.0 ml min^{-1} and the UV absorbance detector was operated at 230 nm. The outlet tubing from the detector was connected to a switch valve to enable automatic fractionation of the eluent. The retention times for methionine, serine, *S*-adenosyl-L-methionine and 4-methylthio-2-oxobutyrate were determined prior to analysis to enable an optimal fractionation. The fractions were collected according to the arrows indicated in Fig. 1 and measured for radioactivity.

2.5. Validation of sample isolation and chromatography

Recovery from isolation was evaluated by addition of L-[methyl-^{11}C]methionine to multiple samples of freshly thawed plasma which was applied to the SCX disposable columns. The columns were washed and the radioactivity retained on the column eluted. Recovery was calculated as the fraction of radioactivity measured in the eluate to the radioactivity in 1 ml plasma, i.e., the volume applied. The selectivity of the isolation procedure for L-[methyl-^{11}C]methionine and in vivo formed metabolites was studied by preparation of a protein-free patient plasma sample using 1 *M* $HClO_4$. The supernatant obtained after centrifugation and filtration (Micro Filtration Systems, Dublin, CA, USA) was injected onto the chromatographic system and the eluate from the detector was collected as described above. The recovery of L-[methyl-^{11}C]methionine and radiolabeled metabolites were compared with those obtained from the automated method with SPE columns. Recovery through the HPLC analysis was determined by comparison of the injected radioactivity to the sum of radioactivity found in the collected fractions.

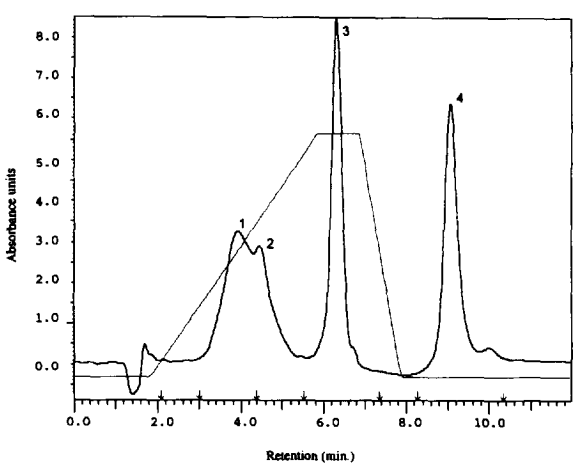

Fig. 1. Chromatogram of L-[methyl-^{11}C]methionine and its metabolites. Peaks: 1=4-methylthio-2-oxobutyrate (0.5 m*M*), 2=L-serine (7.2 m*M*), 3=DL-methionine (0.8 m*M*), 4=*S*-adenosyl-L-methionine (0.2 m*M*). The time events for fractionation are indicated by the arrows.

3. Results and discussion

3.1. Metabolism of L-[methyl-^{11}C]methionine

Methionine is a subject for several metabolic processes in mammalian tissue [5]. Incorporation of L-[methyl-^{11}C]methionine into plasma proteins is reported to be the main route for elimination of L-[methyl-^{11}C]methionine in plasma [7,8]. A minor part of the tracer seems to be metabolised to ^{11}C-labeled serine [7,8] and 4-methylthio-2-oxobutyrate [7]. Furthermore, a third metabolite, ^{11}C-labeled *S*-adenosyl-L-methionine, has been measured in plasma from the rat [2] but has not been detected in human plasma [7]. Consequently, an automated method for determination of the tracer in plasma must include removal of radiolabeled plasma proteins and separation of L-[methyl-^{11}C]methionine from the radiolabeled metabolites.

3.2. Isolation of L-[methyl-^{11}C]methionine from plasma sample

Different methods for isolation of L-[methyl-^{11}C]methionine in plasma samples have previously been used [7,8,11,12]. Size exclusion chromatography (SEC) has been used for separation of radioactivity emanating from the proteins, i.e., the high molecular mass fraction, from the radioactivity in the

low-molecular-mass fraction corresponding to L-[methyl-^{11}C]methionine radiolabeled metabolites [8,11,12]. The radioactivity emanating from the low-molecular-mass fraction was considered to correspond to L-[methyl-^{11}C]methionine and used as reference for the PET quantitation procedure [11]. Hence, there was no correction for possible in vivo-formed radiolabeled metabolites. Despite the error introduced, this value would be preferable to use than total plasma radioactivity.

Another approach was acid precipitation of plasma proteins [7,13]. The supernatant obtained after centrifugation of the sample was introduced onto the HPLC system for separation of radiolabeled methionine and metabolites. Moreover, ultracentrifugation of plasma has been utilised to obtain a sample amenable for HPLC analysis [8]. Solid-phase extraction (SPE) has been performed for determination of the radiotracer concentration in plasma [14]. However, plasma proteins were removed before the sample was applied on the SPE column. None of the methods described were automated and required manual sample handling. Of the methods mentioned, SPE or SEC seem to be the easiest to automate.

In the present study, the plasma sample was directly applied onto the SPE column without any prior procedures. The radioactivity retained on the column after washing needed further analysis to obtain information regarding possible radiolabeled metabolites from L-[methyl-^{11}C]methionine. It was, for this reason, important to avoid residues of proteins in the eluate. However, visual examination of the eluate after addition of $HClO_4$ did not indicate any noticeable amounts of proteins.

Basic, acidic or solutions containing competing cations are examples of eluting solutions which could be used to elute L-[methyl-^{11}C]methionine and radiolabeled metabolites from the SPE column. However, initial experiments proved that NaOH had superior eluting capacity of retained L-[methyl-^{11}C]methionine compared to HCl. Different concentrations (0.1–1 *M*) of NaOH were used to determine the optimal concentration for quantitative elution. Only 10% of the retained radioactivity was eluted when 6 ml of the lowest concentration of NaOH was used but a recovery of over 90% was obtained using 6 ml of NaOH in the concentration range of 0.25–1.0 *M*. Irrespective of concentration, the amino group would become uncharged and the L-[methyl-^{11}C]methionine molecule would dissociate from the negatively-charged sulphonic group on the polystyrene resin. A peak shift with an almost complete recovery of radioactivity found in the first 3 ml fraction of eluate was obvious when the concentration of NaOH increased from 0.25 *M* to 0.6 *M* or higher. The explanation for the increased amount of radioactivity eluted in the first fraction when increasing the concentration of NaOH is probably due to an increased ionic strength of the eluting solution. It was concluded that 3 ml 0.6 *M* NaOH was required to quantitatively elute the retained radioactivity from the SPE column.

3.3. HPLC separation of methionine and metabolites

A capacity of 4 samples per h was desirable using the automated method. Consequently, no more than 8–10 min was allowed for separation of L-[methyl-^{11}C]methionine from metabolites since the time needed for isolation was about 7 min. Moreover, the compartment model used for evaluation of amino-acid transport rate into tumor tissue only requires information on the amount of radioactivity emanating from L-[methyl-^{11}C]methionine compared to the total plasma radioactivity. Hence, the requirement in the analytical method is separation of the radiotracer from its radiolabeled metabolites. Complete resolution among the metabolites is not necessary.

Separation of L-[methyl-^{11}C]methionine from metabolites obtained from biological samples has been performed by both normal-phase [8] as well as ion-exchange liquid chromatography [7]. However, the time needed for separation of L-[methyl-^{11}C]methionine from metabolites in the two systems was in the order of 14–18 min, which hampered their use in the present application. Moreover, separation of both endogenous [15] methionine and ^{14}C-labeled methionine [13] from other amino acids has been performed on reversed-phase columns after derivatisation with *o*-phthaldialdehyde, thereby increasing the normally low retention of methionine on reversed-phase C_{18} columns.

A strong cation-exchange (SCX) chromatography column was used in the present study. High retention of methionine was achieved when the pH of the

mobile phase and sample solution was low. However, methionine was eluted close to the solvent front when the eluate from the SPE column was injected on the chromatographic system. The net charge of the analyte is negative in the basic eluate matrix and might explain the observation. The retention of methionine was considerably increased by acidification of the eluate necessitating a gradient elution to obtain a suitable retention (Fig. 1).

Two proposed metabolites of methionine present in plasma, 4-methylthio-2-oxobutyrate and serine, were both less retained on the SCX column and were completely resolved from methionine. *S*-Adenosyl-L-methionine, although probably not detectable as a radiolabeled metabolite from L-[methyl-^{11}C]-methionine [7], was introduced into the system. The compound was more retained than methionine but complete resolution was still achieved. The total time for separation of methionine from the metabolites was about 10 min (Fig. 1).

3.4. Recovery and precision of the method

The recovery of L-[methyl-^{11}C]methionine in plasma through the SPE procedure was investigated by addition of a small amount of L-[methyl-^{11}C]methionine to 1 ml plasma. The radioactivity recovered in the eluate was 96.1%±1.3 (S.D., n=10) using 500±10 mg of resin and 3 ml eluting solution. The total recovery of L-[methyl-^{11}C]methionine derived radioactivity through the HPLC method was 96.8%±5.0 (S.D., n=20).

A system with minimal changes in retention time of the analytes is extremely important, since the eluent is automatically fractionated. The retention times of the analytes were extremely stable during 8 weeks, even though new mobile phases were prepared and used during this period. The inter- and intra-day variations in retention time were both less than 1.5% (R.S.D.).

3.5. Time considerations for the analysis

Due to the rapid physical decay of the ^{11}C radionuclide, the time factor in the analysis is important. The time needed to process one sample including isolation and separation of L-[methyl-^{11}C]methionine from radiolabeled metabolites was 17 min. However, the time can be shortened by about 2–2.5 min if radioactivity corresponding to ^{11}C-labeled *S*-adenosyl-L-methionine can be neglected (see below). Decreasing the time by about 2.5 min per analysis might not seem that important. However, a reduction of total time for isolation and HPLC procedure by 2.5 min per sample improves the count rate in the fourth sample by about 40%. This clearly shows the impact of how a small gain in time for analysis of a single sample may improve the precision in the assay of the last sample.

3.6. Analysis of patient plasma samples

Samples obtained from patients undergoing PET examinations were analysed using the automated isolation procedure. Large inter-individual variations in the amount of radioactivity found in the fraction retained on the SPE column were observed. This was especially pronounced in samples collected at late time points (Table 1) in agreement with earlier reports [8,9]. The radioactivity in the pass through fraction from the SPE column mainly originates from L-[methyl-^{11}C]methionine incorporated into plasma proteins. No radioactivity was found in the protein fraction after administration of the D-isomer of [methyl-^{11}C]methionine, indicating a selective incorporation of only the L-isomer [8].

Radiolabeled metabolites originating from L-[methyl-^{11}C]methionine appeared in plasma about 10 min after injection of radiotracer into the patient [9]. Radiolabeled serine is one possible metabolite and contributed to about 3% of total plasma radioactivity in man at 30 min after injection of L-[methyl-^{11}C]methionine [7]. Another tentative metabolite of methionine, 4-methylthio-2-oxobutyrate, constituted

Table 1
Fraction of radioactivity retained on the SPE column in samples obtained from patients injected with L-[methyl-^{11}C]methionine at t=0.

Time (min)	Fraction of plasma radioactivity retained on SPE column (%)
5	96± 4
15	88± 4
30	71±13
45	44±13

Each value is the mean±S.D. of 15–25 patients

about 11% of total plasma radioactivity 30 min post-injection of L-[methyl-^{11}C]methionine to patients with tumor disease [7]. In addition, very low concentrations of ^{14}C-labeled *S*-adenosyl-L-methionine have been detected in rat plasma [2]. However, the corresponding ^{11}C-labeled metabolite has not yet been detected in human plasma [7]. The identification of L-[methyl-^{11}C]methionine radiolabeled metabolites has been performed by means of co-elution with a reference compound which cannot be regarded as sufficient evidence for their identity.

Analysis of the plasma samples from three patients using the fully automated method indicated some radioactivity in fractions collected from the HPLC eluent besides that comprising L-[methyl-^{11}C]methionine. The metabolites 4-methylthio-2-oxobutyrate and serine correspond to the collected fractions No. 3 and 4. However, radioactivity was measured in fraction No. 2 corresponding to compounds with no or very low retention. In addition, small amounts of radioactivity was also detected in the third fraction partly corresponding to 4-methylthio-2-oxobutyrate. Radioactivity in these fractions could be detected in the first sample obtained 5 min after injection of the tracer and about 25–35 min onwards. The radioactivity was thereafter not distinguishable from background radioactivity. ^{11}C-labelled *S*-adenosyl-L-methionine was not detectable in the patient plasma samples.

Validation of the selectivity in the isolation procedure with SPE of the automated method was performed by analysis of the composition of L-[methyl-^{11}C]methionine and metabolites in samples from plasma after precipitation of proteins with $HClO_4$. There was no qualitative difference compared with the SPE procedure with respect to metabolite pattern or fraction L-[methyl-^{11}C]methionine of total radioactivity (Table 2). Most of the radioactivity besides L-[methyl-^{11}C]methionine was found in fraction no. 2. The unknown radioactivity constituted about 20% of total radioactivity obtained from the HPLC analysis 25 min after injection of the tracer. Calculated in absolute terms, however, the radioactivity in the collected fraction did not increase with time in some of the patients. This would indicate that the radioactivity found in the second fraction may not be a product of in vivo metabolism of L-[methyl-^{11}C]methionine.

Table 2
Fraction of L-[methyl-^{11}C]methionine derived radioactivity to the total radioactivity after HPLC analysis

Time (min)	Solid-phase extraction (%)	Protein precipitation (%)
5	94± 4 (n=4)	94±1 (n=3)
15	88± 3 (n=3)	87±3 (n=3)
25	82±12 (n=3)	79 (n=2)

Samples are obtained from the automated procedure or after precipitation of plasma proteins using $HClO_4$. Samples are taken from patients at 5, 15 and 25 min after injection of L-[methyl-^{11}C]methionine. Results are expressed as mean±S.D.

The effect of correction of radiolabeled metabolites in plasma on the rate of transport of the tracer into the tumor tissue is shown in Fig. 2. The slope of the line is proportional to the rate and increased about 40% after correction for radiolabeled metabolites. This clearly indicates the necessity of performing analysis of L-[methyl-^{11}C]methionine in plasma during the PET examination.

In conclusion, a fully automated method validated for separation of L-[methyl-^{11}C]methionine and its radiolabeled metabolites in plasma has been developed. The method will be used on routine clinical basis for determination of the fraction of L-[methyl-^{11}C]methionine in plasma to add further precision in the evaluation of tumor radiotracer kinetics measured with PET.

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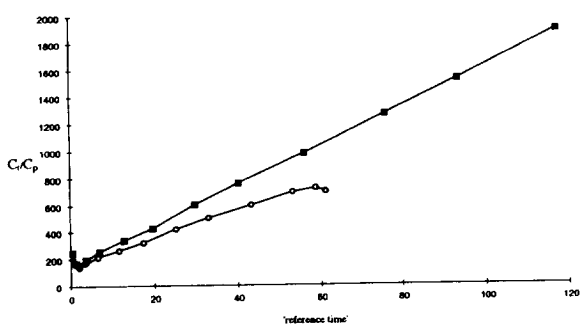

Fig. 2. Calculation of the amino-acid transport rate into tumor tissue with (■) and without (○) correction for L-[methyl-^{11}C]methionine-derived metabolites in plasma. Data is obtained from one patient.

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