Pergamon

0731-7085(95)01308-3

Journal of Pharmaceutical & Biomedical Analysis, Vol. 13, No. 4/5, pp. 353–359, 1995

0731-7085/95 $9.50 + 0.00

An automated liquid chromatographic plasma analysis of amino acids used in combination with positron emission tomography (PET) for determination of *in vivo* plasma kinetics*

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Abstract: Quantification of physiological processes measured with positron emission tomography (PET) requires an "input" function which can be the concentration of administered radio-tracer in plasma. Radioactive nuclides used in PET have short half lives (2–20 min) and a limited time is available for the PET investigation including analysis of the composition of the radioactive signal in plasma. Therefore, an automated method for the analysis and separation of β-[^{11}C]-L-5-hydroxy-tryptophan ([^{11}C]-L-5-HTP), β-[^{11}C]-L-3,4-dihydroxy-phenylalanine ([^{11}C]-L-DOPA) and L-[methyl-^{11}C]-methionine and their respective metabolites in plasma was developed. A size exclusion column was used for isolation of the low molecular weight fraction. In the case of [^{11}C]-L-5-HTP and [^{11}C]-L-DOPA, the low molecular weight fraction was injected onto a liquid chromatographic system for separation of radioactive tracer from *in vivo* formed radio-labelled metabolites. The elution volume from the size exclusion column was 7.0, 5.0 and 3.5 ml for [^{11}C]-L-5-HTP, [^{11}C]-L-DOPA and L-[methyl-^{11}C]-methionine, respectively. An interaction with the column matrix and the solutes was observed for both [^{11}C]-L-5-HTP and [^{11}C]-L-DOPA. The yield in the isolation step was >98%. Separation of [^{11}C]-L-5-HTP and [^{11}C]-L-DOPA from their respective metabolites was performed with high-performance liquid chromatography with automated collection of fractions of the eluate corresponding to those of administered tracer and metabolites. The fractions were measured for radioactivity in a well counter. Inter- and intraday variation in retention were less than 3% RSD. Fortyfive minutes after injection of [^{11}C]-L-5-HTP or [^{11}C]-L-DOPA the radioactivity in the [^{11}C]-L-5-HTP or [^{11}C]-L-DOPA fraction was 58.5 ± 6.4 (RSD, n = 4) and 31.0 ± 2.7 (RSD, n = 3), respectively. High and low molecular weight fractions from plasma after injection of L-[methyl-^{11}C]-methionine were collected and measured for radioactivity. An incorporation of [^{11}C]-L-methionine in plasma proteins, i.e. the high molecular weight fraction, was observed but with large inter-individual differences.

Keywords: *Amino acids; liquid chromatography; plasma analysis; positron emission tomography.*

Introduction

Fully automated analytical procedures comprising isolation, liquid chromatographic separation and quantitation of analyte and metabolites are highly requested particularly in studies of large number of biological samples. Analysis of radio-labelled compounds in plasma samples also requires automated methods in order to increase speed and reproducibility of quantitation as well as protection of personnel from radiation hazards. This approach is of particular importance in the analysis of radio-labelled compounds with short physical half-life as e.g. used in clinical applications with positron emission tomography, PET. Typically radionuclides used in PET are ^{15}O, ^{13}N, ^{11}C with a half-life of 2.07, 9.97 and 20.3 min, respectively.

PET is a non-invasive tracer technique which enables quantitation of the kinetics of physiological processes in the tissue of living animal or man. Processes studied are diagnosis of disease and evaluation of treatment effects which include measurement of blood flow and blood volume in the tissue, energy utilization

*Presented at the Fifth International Symposium on Pharmaceutical and Biomedical Analysis, Stockholm, Sweden, September 1994.

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in the heart and brain, protein synthesis as well as quantitation of enzyme kinetics and characterization of binding of drugs to different receptor types [1]. Radiolabelling is performed with short-lived radionuclides such as ^{11}C, ^{15}O and ^{13}N giving radio-tracers with chemical properties similar to endogenous compounds or drugs. Quantitation of the kinetics of the process under study relies on the "input" function to the tissue which usually is the administered radio-tracer concentration in plasma. This requires that the radioactive constitution of plasma is determined. An extensive metabolism of radio-labelled endogenous compounds requests an on-line blood or plasma analysis of radio-tracer separated from formed radio-labelled metabolites. The short physical half-life of radio-tracers thus requires very fast, robust and simple analytical methods with high analysis capacity. Metabolite analysis is also wanted although the metabolic rate is low since the volume of distribution of the formed radio-labelled metabolites might be much less as compared to the parent radio-tracer.

[^{11}C]-L-5-HTP and [^{11}C]-L-DOPA are used with PET in studies of psychiatric [2] and neurological [3] diseases but are also markers for localization, diagnosis and validation of treatment effects of neuroendocrine tumours [4]. Adequate quantitation of tumour growth and treatment response required knowledge of radio-tracer concentration in the blood without interference from radio-labelled metabolites.

In this study, an automated analytical method is presented for the analysis of [^{11}C]-L-5-HTP and [^{11}C]-L-DOPA as well as L-[methyl-^{11}C]-methionine and *in vivo* formed radio-labelled metabolites. The system consists of a laboratory robot comprising separation of analytes from plasma proteins using size exclusion chromatography followed by separation of low molecular weight components by reversed-phase liquid chromatography and quantitation of radioactivity in fractions of the eluate.

Experimental

Apparatus

For the separation of high and low molecular weight fractions, a size exclusion chromatography column, HiTrap™ Desalting (Pharmacia Biotech, Sollentuna, Sweden), was used. The fractionation range of the column was 1000–5000 D with a cut off of approximately 5000 D. The flow through the column was controlled by an automated solid phase extraction controller (ASPEC, Gilson, Middleton, WI, USA) with a dilutor (model nr 401, Gilson). The low molecular weight fraction was collected and injected onto a chromatographic system consisting of a dual pump system (model nr 306, Gilson), a mixer and a UV-detector (model nr UV 117, Gilson) operated at 254 nm. The chromatographic system was controlled by a computer with a Gilson 715 HPLC software. Fractions according to the corresponding UV-signal were automatically collected by the ASPEC device. The radioactivity was measured manually in a well type scintillation counter built in house (NaI (Tl) crystal [3 × 3 in, 2 in depth and 28 mm i.d.], equipped with an amplifier, low discriminator and a scaler).

Chemicals and reagents

Reference compounds (DL-β-3,4-dihydroxy-L-phenylalanine [DL-DOPA], 3-O-methyl-L-DOPA [3-O-MD], dopamine [DA], homovanillic acid [HVA], 3,4-dihydroxy-phenylacetic acid [DOPAC], serotonin [5-HT] and 5-hydroxyindol-3-acetic acid [5-HIAA]) were obtained from the Sigma Chemical Company (St Louis, MO, USA). 5-Hydroxy-L-tryptophan [5-HTP] was obtained from Aldrich-Chemie (Steinheim, Germany). Trifluoroacetic acid [TFA], acetic acid and acetonitrile were purchased from Merck (Darmstadt, Germany). Saline 9 mg ml^{-1} was obtained from Kabi Pharmacia (Uppsala, Sweden). Purified water (NANOpure, Barnstead, Iowa, USA) was used for preparation of mobile phases.

The radionuclide [^{11}C] was produced in a Scanditronix M17 cyclotron, Uppsala University PET centre, and obtained as [^{11}C]-carbon dioxide which was used in a series of organic chemical and enzymatic synthesis steps to produce [^{11}C]-L-DOPA or [^{11}C]-L-5-HTP radio-labelled in the metabolic stable β-position, regarding decarboxylation by aromatic L-amino acid decarboxylase, (L-AADC, E.C. 4.1.1.28) [5, 6]. L-[methyl-^{11}C]-methionine was synthesized via methylation of the precursor S-benzyl-L-homocysteine [7]. After identification and control of radiochemical purity the preparation was filtered through a 0.22-μm filter before administration. The radioactive dose

administered intravenously to patients varied between 200 and 800 MBq.

Sample preparation

The blood samples were collected in glass tubes and centrifuged at 4000 rpm for 4 min. Of plasma, 1.7 ml was transferred to an Eppendorf tube and placed in the ASPEC before start of analysis. Of plasma, 1.5 ml was applied to the size exclusion chromatography column. Plasma containing [^{11}C]-L-DOPA or [^{11}C]-L-5-HTP and their respective metabolites was eluted with water. The low molecular weight fraction was collected and a standard solution containing 5-HTP or L-DOPA and their respective metabolites was added to a final concentration of 50–80 μM before further analysis on the HPLC system. L-[methyl-^{11}C]-methionine, metabolites and L-[methyl-^{11}C]-methionine incorporated into proteins, were eluted with saline. The flow of the mobile phase through the size exclusion chromatography column was 6 ml min^{-1}.

Liquid chromatography

The low molecular weight fraction was injected via a loop of 550 μl onto the chromatographic system. Separation of [^{11}C]-L-DOPA or [^{11}C]-L-5-HTP and metabolites was performed on a C-18 column (YMC Pack ODS-AL, 100 × 4.6 mm i.d., 5 μm particle size, YMC Co. Ltd, Kyoto, Japan). The separation of administered compound from metabolites was performed with an acidic aqueous mobile phase and an organic modifier, acetonitrile, in the gradient. The mobile phase for separation of [^{11}C]-L-DOPA and metabolites contained acetic acid (10 mM, pH 3.3). For the separation of [^{11}C]-L-5-HTP and metabolites a slightly higher concentration of acetic acid was used (17 mM, pH* 3.3) with addition of 10 mM TFA. The chromatographic conditions are summarized in Table 1.

Validation of the size exclusion chromatography column and HPLC procedure

After application of the plasma was, the size exclusion chromatography column was disconnected from the ASPEC device and placed in the well counter for measurement of the radioactivity. Thereafter, the column was reconnected, eluted and once again disconnected for measurement of the remaining radioactivity. The yield through the isolation step was then calculated as the fraction of remaining radioactivity to the applied radioactivity. The retention volume of [^{11}C]-L-5-HTP, [^{11}C]-L-DOPA and L-[methyl-^{11}C]-methionine from the size exclusion chromatography column was determined by continuously collecting the eluate in fractions of 0.5 ml for measurement of radioactivity. An elution profile for each tracer was constructed (Fig. 1) from the data obtained. The fractions corresponding to radioactivity in the low molecular weight range, i.e. tracer amino acid and radio-labelled metabolites, were pooled and injected to the HPLC system. After validation of the isolation step the procedure was automated and only two fractions, the high and low molecular weight fraction, were collected.

The yield through the HPLC analysis was obtained by comparing the radioactivity concentration in the sample solution injected for metabolite analysis to the total radioactivity eluted from the HPLC-column.

Results

Isolation of analyte

The retention volumes in the size exclusion chromatography column of [^{11}C]-L-5-HTP and [^{11}C]-L-DOPA derived radioactivity were 7

Table 1
Mobile phase composition for separation of [^{11}C]-5-HTP or [^{11}C]-l-DOPA and respective metabolites

Analyte	Mobile phase	Gradient (% Acetonitrile)	
[^{11}C]-L-5-HTP	17 mM Acetic acid 10 mM TFA pH 3.3	Start (t = 0 min) t = 4–8 min t = 8–9 min t = 9–10 min	6% 6–100% 100% 100–6%
[^{11}C]-L-DOPA	10 mM acetic acid pH 3.3	Start (t = 0 min) t = 2.5–8.0 min t = 8–9 min t = 9–10 min	0% 0–100% 100% 100–0%

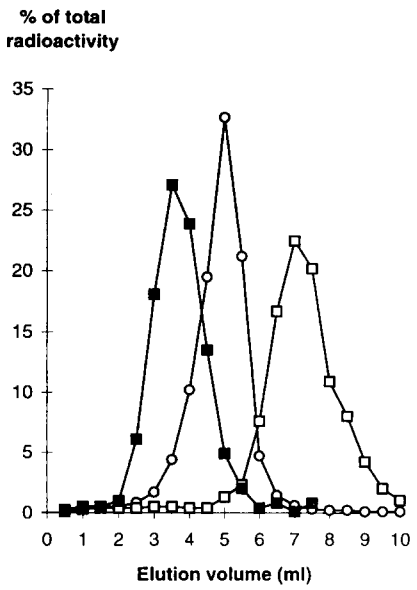

Figure 1
Elution profile of [^{11}C]-L-5-HTP (□), [^{11}C)-L-DOPA (○) and L-[methyl-^{11}C]-methionine (■) from HiTrap™ Desalting column. The plasma sample was obtained 45 min after injection.

and 5 ml, respectively (Fig. 1). The retention volume for L-[methyl-^{11}C]-methionine was only 3.5 ml (Fig. 1). The pore volume of the matrix was determined to 3.5 ml by means of acetone as a marker. The yield through the pre-purification step was 98.4% (RSD = 1.1%, n = 8) and 99.6% (RSD = 0.2%, n = 5) for [^{11}C]-L-5-HTP and [^{11}C]-L-DOPA, respectively, with an elution volume of 11.25 ml water. A yield of 99.6% (RSD = 0.5%, n = 8) was obtained for L-[methyl-^{11}C]-methionine when the column was eluted with 7.5 ml saline.

Separation and quantitation

Separation of the radio-tracer and the radioactivity derived from formed metabolites was achieved using a C-18 column with similar mobile phases and elution programs for samples containing [^{11}C]-L-5-HTP (Fig. 2) or [^{11}C]-L-DOPA (Fig. 3). Trifluoroacetic acid was added to improve the resolution between [^{11}C]-L-5-HTP and the labelled metabolites. The chromatographic analysis was performed during 13 min including time for re-equilibration. Fractions determined from the UV-signal of the compounds was collected and measured for radioactivity. The radioactivity in the fraction corresponding to [^{11}C]-L-5-HTP and [^{11}C]-L-DOPA decreased with time. Between 40 and 50 min after injection, about 60 and 30% of the eluted radioactivity was found in the fractions of [^{11}C]-L-5-HTP and [^{11}C]-L-DOPA, respectively (Table 2).

The total time required for a complete analysis of four samples was 72 min, including sample-workup, HPLC-analysis and reconditioning of the HiTrap™ Desalting and HPLC column. The absolute yield through the HPLC analysis was 98.1% (RSD = 7.0%, n = 10). The intra- and interday variation in the retention of [^{11}C]-L-5-HTP and [^{11}C]-L-DOPA in the chromatographic system was less than 3% RSD.

Discussion

The calculation of an estimate of growth and treatment response of neuroendocrine tumors using PET with [^{11}C]-L-5-HTP nad [^{11}C]-L-DOPA as radio-tracers required quantitation of the administered tracer in plasma. Determination of the fraction of unmetabolized tracer was therefore necessary to perform if the models used in the quantitation process is to be used in an optimal manner. The developed methodology should be fully automated in order to ensure reproducibility, speed and radiation protection to personnel. The present fully automated method fulfilled the requirements of four complete analysis in 72 min with high intra- and interassay precision. The time needed for four complete analysis can however be reduced in many parts, for example by increasing the flow through the HiTrap™ Desalting column and/or decreasing the time for separation of tracer and the *in vivo* formed radio-labelled metabolites by optimizing the mobile phase as well as the profile of the gradient or use of shorter columns in combination with a reduced particle size. The total time for analysis could probably be reduced to about 60 min. Using these short-lived radionuclides a small gain in time for analysis would dramatically increase the count rate of the sample. An example will show this fact. A half-life of 20.3 min and an assumed radioactivity of 100 counts per second (cps) measured after 72 min, the radioactivity 12 min earlier, i.e. at 60 min, will result in a count rate of 151 cps. Hence, by reducing the total time of analysis with 12 min, the number of counts measured in each peak measured would increase more than 50%. This clearly indicates the importance of reducing the total time for analysis.

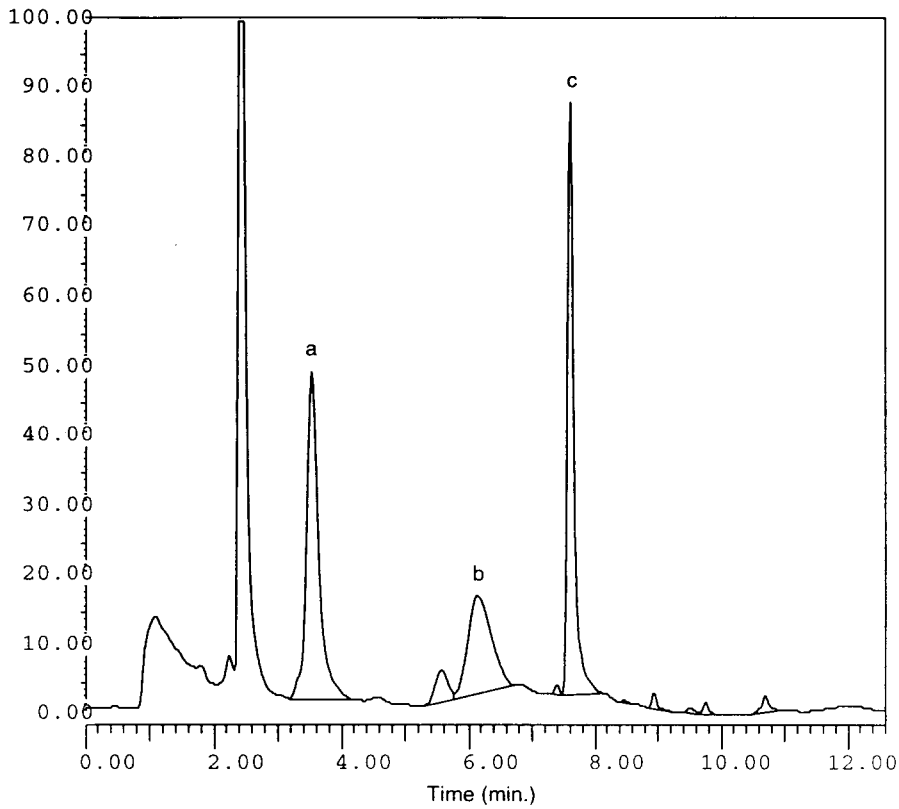

Figure 2
Separation of 5-HTP, 5-HT and 5-HIAA using HPLC. The sample was obtained after isolation of the [^{11}C]-derived radioactivity using size exclusion chromatography. To the sample a reference solution was added to a final concentration of 60–80 μM before injection into the chromatographic system. Key to peaks (min): (a) 5-HTP:t_r, 3.6; (b) 5-HT:t_r, 6.2; (c) 5-HIAA:t_r, 7.8.

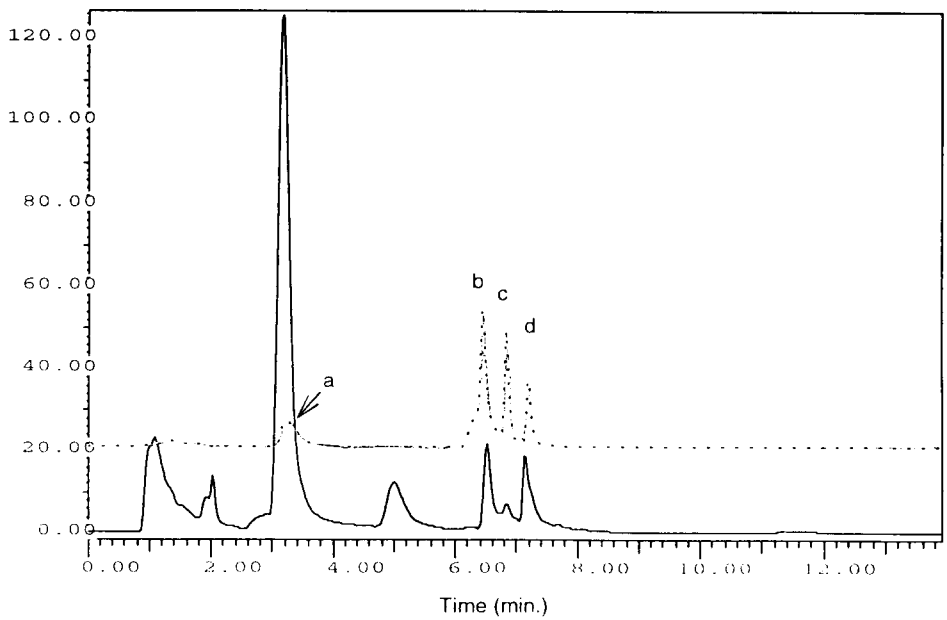

Figure 3
Chromatograms from authentic sample (lower) and standard solution (upper) showing separation of L-DOPA and metabolites. The sample was obtained after isolation of the [^{11}C]-derived radioactivity using size exclusion chromatography. A reference solution was added to the before injection into the chromatographic system. Final concentration was 50–70 μM. Key to peaks (min): (a) DL-DOPA:t_r, 3.3; (b) DA + Me-DOPA:t_r, 6.5; (c) DOPAC:t_r, 6.9; (d) HVA: 7.3.

Table 2
Analysis of [^{11}C]-L-5-HTP and [^{11}C]-L-DOPA in plasma during 45 min. Results are expressed as % ±SD of total radioactivity obtained in the eluate from the HPLC analysis

Time (min)	[^{11}C]-L-5-HTP (n = 4)	[^{11}C]-L-DOPA (n = 3)
5	86.4 ± 6.5	82.6 ± 7.4
25	70.0 ± 5.6	37.7 ± 3.2
45	58.5 ± 6.4	31.0 ± 2.7

The current method has been in routine use for clinical assessment on neuroendocrine tumors and added precision to the quantitation using PET as compared with assessment without correction for metabolites formed during the investigation.

Many different methods have been presented for the analysis of endogenous levels of indole and catecholamines from biological samples. Most of the methods include a manual step for removing plasma proteins either with addition of acid or organic solvent [8, 9] or ultracentrifugation [10]. These methods are however difficult to fully automate. Other methods include solid phase extractions on either polystyrene materials [11], C-18 [12] or protein coated ODS [13] columns. Methods comprising a solid phase extraction column are easy to automate. However, a drawback is that an evaporation of the organic modifier used in most methods for the elution of the hydrophobic substances of interest. This step can be difficult to integrate in an automated method. Coupled columns have been used for direct chromatographic analysis of plasma samples without preceding separation of plasma proteins but the method used a three-pump system with three six port valves and three columns [14]. Microdialysis was also employed to remove plasma proteins in the analysis of indolamines and catecholamines in order to achieve a sample amenable for direct injection into the liquid chromatographic system [15]. The use of dialysis or ultrafiltration as a pre-purification step is for this application, however, probably not useful, since the time to achieve a sample containing radioactivity enough for further analysis might be too long. A size exclusion column, HiTrap™ Desalting, was used in the present robotic controlled procedure for isolation of the radio-tracer amino acid together with formed radio-labelled metabolites. The compounds of interest were quantitatively fractionated in about 6 ml of the eluate. In the case of [^{11}C]-L-5-HTP and [^{11}C]-L-DOPA a single, relatively broad radioactivity peak was observed corresponding to a low molecular weight fraction. Different retention volumes for the tracer and metabolites through the column could not be excluded. Therefore, the whole peak had to be collected and a fraction of it was then used in the subsequent HPLC analysis.

An obvious interaction of the radioactive material with the column matrix occurred for [^{11}C]-L-5-HTP and [^{11}C)-L-DOPA since found retention volumes were larger than the calculated pore volume. Possible interactions are of ionic, aromatic and hydrophobic character. Hydrogen bonding between the matrix and solute, however, is also a possible interaction. The eluents used were water or solutions of sodium chloride which should suppress interactions of the hydrogen bonding type. Increasing the ionic strength to 1 M sodium chloride did not decrease the elution volume which was expected if the interaction was of the ionic type. Contradictory, a slightly increased elution volume was instead observed, indicating a possible hydrophobic interaction. Hydrophobic interactions have been reported for tryptophan eluted on a Sephadex® G-25 matrix [16]. The interaction can however be favorable since the separation of high and low molecular radioactivity weight fractions is increased thereby reducing the risk of injecting small proteins on the analytical separation column. The pre-purification step provided fast, reliable and a fully automated isolation step of [^{11}C]-L-DOPA or [^{11}C]-L-5-HTP and metabolites from the plasma proteins. The HiTrap column was also proved to be useful in routine analysis for the separation of unbound L-[methyl-^{1}C]-methionine including low molecular weight metabolites and L-[methyl-^{11}C]-methionine incorporated into different plasma proteins. Large inter-individual differences in metabolism of L-[methyl-^{11}C]-methionine was observed (Fig. 4) indicating the necessity of analysis of the fraction of unbound L-[methyl-^{11}C]-methionine. The elution volume of L-[methyl-^{11}C]-methionine was 3.5 ml indicating no major interactions since it corresponded to the total pore volume of the matrix (data not shown).

The kinetic model used for the quantitation of the utilization [^{11}C]-5-L-HTP and [^{11}C]-L-DOPA in the clinical PET procedure only

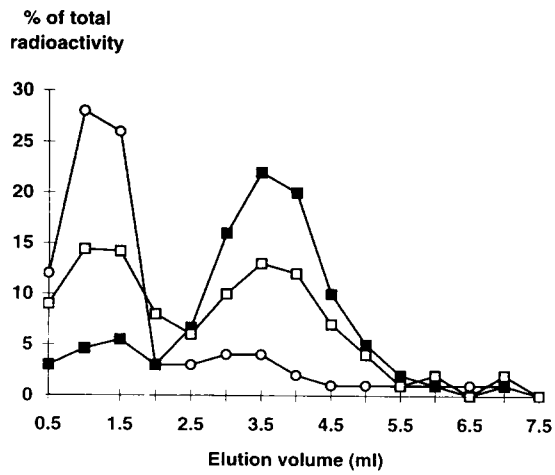

Figure 4
Elution profile of L-[methyl-^{11}C]-methionine from HiTrap™ Desalting column. The plasma sample was obtained 45 min after injection. The symbols (□), (○) and (■) represents three different patients.

required a separation of the radioactivity emanating from parent compound and the sum of radio-labelled metabolites, respectively. This made possible the application of a gradient of the mobile phase after elution of the radio-labelled tracer molecule and collection of all radio-labelled metabolites in one fraction eluting after some 8–10 ml. However, in the chromatographic system used for separation of [^{11}C]-5-L-HTP and labelled metabolites TFA was added to improve resolution. The reason for this was that the chromatographic system was also developed and used for other applications where complete resolution between the two labelled metabolites, [^{11}C] labelled 5-HIAA and 5-HT, was necessary. A complete separation of the two fractions containing tracer and metabolites, respectively, guaranteed the selectivity of the method since other compounds of endogenous origin co-eluting with the radioactive components was not able to contribute to the measured radio-signal. Radio-detection has a high sensitivity and is well compatible with other methods of detection of catecholamines as e.g. UV-absorption, fluorescence or electrochemical detection.

Conclusion

Due to a rapid metabolism in the plasma of [^{11}C]-L-5-HTP, [^{11}C]-L-DOPA and L-[methyl-^{11}C]-methionine, the quantitation of tumour growth and response after medical treatment measured with PET using compartmental models with total plasma radioactivity as reference tissue, is a source of error in the quantification process. A fully automated chromatographic method has been implemented for routine use in clinical tumour investigations giving reliable, fast and accurate estimations of radio-tracer and metabolite concentrations in the reference tissue.

Acknowledgement — This study was financially supported by the Swedish Medical Research Council, grant no. 08645.

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[*Received for review 12 October 1994; revised manuscript received 21 November 1994*]