Pergamon

0731-7085(95)01291-5

Journal of Pharmaceutical & Biomedical Analysis, Vol. 13, No. 4/5, pp. 361–367, 1995
Elsevier Science Ltd
Printed in Great Britain
0731-7085/95 $9.50 + 0.00

Liquid chromatographic analysis of brain homogenates and microdialysates for the quantification of L-[β-^{11}C]DOPA and its metabolites for the validation of positron emission tomography studies

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Abstract: The clinical use of positron emission tomography, PET, with selected radiolabelled tracer molecules visualizing and quantitating physiological processes in the tissue relies in many situations on compartmental models for the interpretation of the radiosignal. Validation of such models must, therefore, include chromatographic analysis of the radioactivity composition of the signal. Rapid and sensitive liquid chromatographic methods amenable for automation for the analysis of [^{11}C] labelled L-DOPA and its metabolites were therefore developed and validated for the quantitation of radioactivity composition in rat brain microdialysates as well as homogenates. Analysis included a simple isolation step, separation using reversed phase liquid chromatography with radiometric detection and permitted assay following tracer doses with an analysis time of 15 min. The analysis of radioactivity composition in the rat striatum showed that peripherally formed *O*-methyl L-DOPA constituted less than 20% of the radioactivity 40 min after injection of L-[β-^{11}C]DOPA. In the extracellular space the main component was [^{11}C]-homovanillic acid which increased with time indicating rapid formation but slow elimination. The cumulation of radioactivity in the striatum corresponded to the radioactivity signal of dopamine and derived metabolites. The formation rate of dopamine in the rat corresponded closely to the utilization rate in the striatum of monkey and man measured with PET. This indicated that the rate constants measured with PET correlates well to the dopamine synthesis rate.

Keywords: *L-[β-^{11}C]DOPA; dopamine synthesis rate; positron emission tomography; microdialysis; rat brain homogenate.*

Introduction

Positron emission tomography allows *in vivo* quantitation of radiotracer kinetics in a physiological and biochemical process. Synthesis of endogenous substances radiolabelled with short-lived radionuclides ^{11}C ($t_{½}$ = 20.3 min), ^{13}N ($t_{½}$ = 9.97 min) and ^{15}O ($t_{½}$ = 2.07 min) offers kinetic information regarding processes such as energy utilization, blood flow, receptor binding, enzyme activity and drug transport. The kinetics is calculated using compartment models employing the radioactivity change over time measured in the tissue. However, the radioactivity signal emanates both from administered radiotracer as well as from formed radiolabelled metabolites. To validate quantitative measurements of such processes, independent methods for their determination are required. Rapid and sensitive liquid chromatographic methods amenable for automation may, therefore, be useful.

It has been shown that regional brain neurotransmitter synthesis of dopamine was up-

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

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regulated in advanced Parkinson's disease [1] and of serotonin in major depression [2]. Neurotransmitter synthesis rate has been quantitated using PET with L-[β-^{11}C]DOPA [3, 4] or [^{11}C]-5-hydroxy-L-tryptophan [3, 5] as tracers, both radiolabelled in the metabolic stable β-position. However, an altered radioactive signal from the tissue did not necessarily have to correlate to an altered synthesis rate. Therefore, selective blocking of individual enzymes [3, 6] and multipositional labelling [4, 6] was used to indicate that the decarboxylation rate of the tracer was measured. A negligible radioactivity contribution from peripherally formed metabolites was assessed in rat brain homogenates [7, 8].

However, to further validate the quantification of kinetic parameters with PET, determination of the intra- and extracellular distribution in the brain of [^{11}C]-radiolabelled components was required. In the present study, high-performance liquid chromatography was used to measure concentrations of L-[β-^{11}C]-DOPA and its radiolabelled metabolites in rat brain microdialysates and homogenates. In addition, specific brain cumulation of L-[β-^{11}C]DOPA and radiolabelled metabolites in various brain areas enabled measurement of brain synthesis rate of [^{11}C]-dopamine ([^{11}C]-DA) which was compared with the rate constant for L-[β-^{11}C]DOPA utilization measured in the striatum of monkey [3, 9] and man [1] using PET.

Materials and Methods

Synthesis of L-[β-^{11}C]DOPA

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 L-[β-^{11}C]DOPA radiolabelled in the metabolic stable β-position [10]. After identification and control of radiochemical purity the preparation was filtered through a 0.22-μm filter before administration. The radioactive dose was administered intravenously and varied between 108 and 388 MBq corresponding to an injected amount of L-DOPA of 1–16 μg.

Chemicals

3,4-Dihydroxy-phenyl-L-alanine (L-DOPA); dopamine (DA), homovanillic acid (HVA); 3-O-methyl-L-DOPA (Me-DOPA), 3,4-dihydroxy-phenylacetic acid (DOPAC) and sodium bisulphite were obtained from the Sigma Chemical Company (St Louis, MO, USA). Perchloric acid solution (PCA), buffers and organic solvents were of analytical grade and purchased from Merck (Darmstadt, Germany). EDTA was obtained from KEBO AB (Stockholm, Sweden), whereas 1-heptanesulphonic acid as the sodium salt, was purchased from Aldrich-Chemie (Steinheim, Germany).

Animals

Male Sprague Dawley rats (ALAB, Sollentuna Sweden) weighing 290–395 g were used. They were housed in cages with a 12 h dark/light cycle and with free access to food and water. The animals were allowed to recover from transport for at least one week. Anaesthesia was induced with intraperitoneal chloral hydrate (Apoteksbolaget, Stockholm, Sweden), 450 mg kg^{-1} and maintained by constant rate infusion of 100–150 mg kg^{-1} h^{-1} through a catheter inserted into the femoral vein of a hind leg.

All experiments were performed with permission from the local Research Animal Ethics Committee.

Microdialysis

The animal was placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). A microdialysis probe (CMA 12, CMA Microdialysis AB, Stockholm) with a 3-mm membrane was inserted into the right striatum according to the atlas of Paxino (L: 3 mm, A: 0 mm, V: 6.5 mm). The probe was perfused at 5 μl min^{-1} (CMA 100 microinjection pump, CMA Microdialysis AB) with a modified Ringer solution (Apoteksbolaget, Umeå, Sweden). Samples were collected every 10 min in tubes containing 5 μl PCA (1 M) with EDTA (1 mM). Forty minutes after the radioactive dose the rats were killed and the brain was quickly removed and placed on ice where the striatum and the cerebellum were dissected. The tissues were weighted and the radioactivity in the tissue measured in a well-counter.

Preparation of tissue samples and analysis of L-[β-^{11}C]DOPA derived radioactivity

Brain tissue was homogenized (Polytron PT 3000, Kinematica AG Littau, Switzerland) in

1 ml cold PCA (0.1 M) containing sodium bisulphite (1 mg ml^{-1}) and EDTA (1 mM). The homogenate was centrifuged at 12 000 rpm for 2 min (Heraeus Biofuge 15, Heraeus Sepatech GmBh, Osterode, Germany). The supernatant was isolated, filtered through a 0.22 μm cellulose acetate membrane filter (Nalgene, NY, USA). The supernatant (0.8 ml) was spiked with 30 μl of a standard solution containing 2 mM of each L-DOPA/Me-DOPA, DOPAC, DA and HVA before injection to the chromatographic system.

L-[β-^{11}C]DOPA and radiolabelled metabolites were separated using reversed-phase liquid chromatography. One hundred microlitres of the sample was injected onto the HPLC system. The chromatographic system consisted of a pump (Gilson 306, Middleton, WI, USA), a dynamic mixer (model No. 811C, Gilson) and a UV-detector (model No. UV 117, Gilson) operated at 254 nm. The liquid chromatograph was connected to an automated solid phase extraction controller (ASPEC, Gilson) used as a fraction collector. Fractions were collected automatically and the radioactivity in each collected fraction was measured. The mobile phase consisted of a citric acid-sodium acetate buffer (0.1 M)-EDTA (1 mM)-sodium heptanesulphonate (4.5–5.3 mM)–methanol (87.5:12.5, v/v) (pH* 3.7). The separation was performed on a C-18 column (BAS Biophase ODS II-3 μm 150 × 4.6 mm i.d., BAS, West Lafayette, IN, USA). Before each experiment, the retention times for L-DOPA, Me-DOPA, DOPAC, DA and HVA were checked and the time window for collecting the fractions containing the desired peaks adjusted accordingly.

The yield through the procedure was calculated as the total radioactivity eluted from the column to the total radioactivity in the dissected tissue before homogenization.

Analysis of L-[β-^{11}C]DOPA-derived radioactivity from microdialysates

The separation of L-[β-^{11}C]DOPA and radiolabelled metabolites in microdialysates was performed with a liquid chromatograph equipped with electrochemical detection. The system consisted of a pump with a flow of 280 μl min^{-1} (BAS PM60), a column oven kept at 37.5°C by use of a thermostatic temperature control (BAS LC22) and an electrochemical detector (BAS LC4C). The oxidation potential was set at +0.7 V against an Ag/AgCl reference electrode. Forty microlitres of the dialysates was directly injected (CMA 200, CMA Microdialysis AB) onto the column (BAS Biophase ODSII-3 μm 150 × 2 mm i.d.). The composition of the mobile phase has been described elsewhere [11], but had to be slightly modified to contain 0.83 mM octanesulphonic acid. The fractions containing L-[β-^{11}C]DOPA, [^{11}C]-DA, [^{11}C]-Me-DOPA, [^{11}C]-DOPAC and [^{11}C] were collected manually and counted for radioactivity in a well-counter.

Calculations

The radioactivity was measured in a well-type scintillation counter for 90 s and corrected for physical decay to the time of administration of the radioactive dose. All results are expressed as "uptake", i.e. the concentration of radioactivity in the collected fraction normalized to the weight of the dissected tissue and injected radioactive dose per gram bodyweight.

The rate constant for synthesis of [^{11}C]-DA from L-[β-^{11}C]DOPA was calculated using two methods. The first was similar to the calculation used with PET in monkey [3] and man [1], utilizing a graphical analysis with a brain reference tissue as input function and linearizing the uptake of L-[β-^{11}C]DOPA into the striatum. The slope was assumed to be proportional to the rate constant of the unidirectional flux of radioactivity to the striatum for decarboxylation. The rate constant was also calculated from the fraction of radioactivity in the striatum giving [^{11}C]-dopamine and its radiolabelled metabolites, simply by dividing the total uptake with time (see Table 1).

Table 1
Normalized radioactivity uptake in the L-[β-^{11}C]DOPA and metabolite fractions 40 min after injection of L-[β-^{11}C]DOPA. The supernatant from the striatum and cerebellum homogenate was analyzed using HPLC as described in "Materials and methods". Each value represents the mean for three animals ± SD

Region	[^{11}C]-Dopa	[^{11}C]-Me-Dopa	[^{11}C]-DOPAC	[^{11}C]-DA	[^{11}C]-HVA
Striatum	0.0210 ± 0.001	0.0161 ± 0.003	0.0127 ± 0.0001	0.0234 ± 0.0051	0.0225 ± 0.0046
Cerebellum	0.0088 ± 0.0007	0.0134 ±0.0021	0.0037 ± 0.0014	0.0017 ± 0.0007	0.0229 ± 0.0073

Results

Liquid chromatographic analysis of L-[β-^{11}C]-*DOPA derived radioactivity*

To reduce total time for analysis two parallel chromatographic systems were used. Complete resolution of the components was obtained within 10–14 min allowing analysis of four to six samples every hour. L-DOPA eluted in both systems close to the front. To reduce the retention of HVA an organic modifier had to be added, thereby reducing the already short retention of L-DOPA. In the microdialysates no endogenous levels of L-DOPA and 3-*O*-

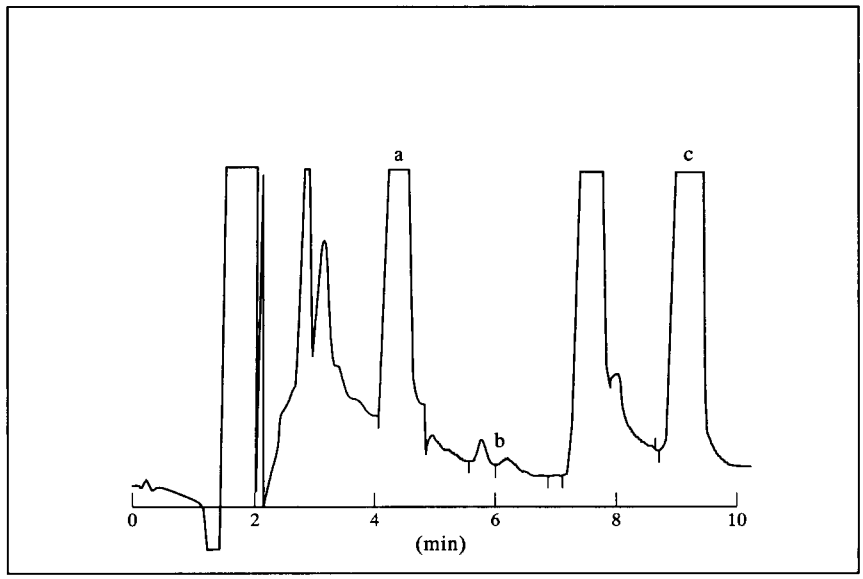

Figure 1
Chromatogram obtained after injection of a microdialysate. Endogenous levels of L-DOPA and 3-*O*-methyl-L-DOPA were below the detection limit. Key to peaks (minutes): (a) DOPAC: t_R, 4.2; (b) DA: t_R, 6.1; (c) HVA: t_R, 9.0. The retention times of L-DOPA and 3-*O*-methyl-L-DOPA were 2.0 and 2.7 min, respectively, according to a standard solution.

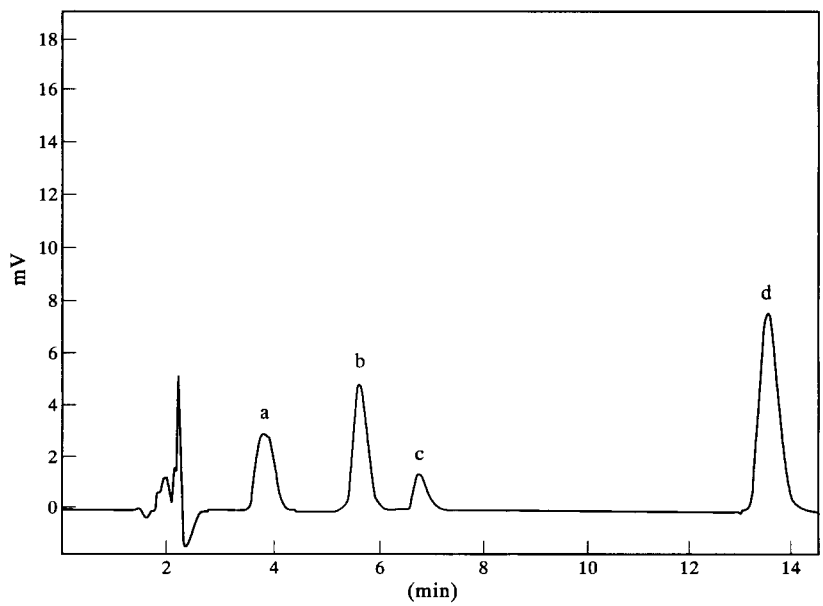

Figure 2
Chromatogram obtained after injection of a striatum homogenate. A standard solution containing L-DOPA, 3-*O*-methyl-L-DOPA, DA, DOPAC and HVA was added to the supernatant. The final concentration was 0.08 mM. Key to peaks (min): (a) 3-*O*-methyl-L-DOPA: t_R, 3.8; (b) DOPAC: t_R, 5.6; (c) DA: t_R, 6.8; and (d) HVA: t_R, 13.6, L-DOPA eluted close to the solvent front, t_R 2.2 min.

methyl-L-DOPA were found while DA, DOPAC and HVA were detected (Fig. 1). UV detection was employed for the analysis of homogenates. The endogenous levels in the homogenate were too low to detect. A standard solution was added to the supernatant to visualize the peaks to ensure that a correct fractionation was done (Fig. 2).

Brain disposition of L-[β-^{11}C]DOPA derived radioactivity

Brain tissue samples. Forty minutes after administration of the radioactive dose 22% of the radioactivity in the striatum corresponded to unchanged L-[β-^{11}C]DOPA, whereas 61% of the radioactivity was found in the [^{11}C]-DA, [^{11}C]-HVA and [^{11}C]-DOPAC fractions. [^{11}C]-Me-DOPA comprised of some 17% of the radioactivity. In the cerebellum similar uptake values as in the striatum were measured for [^{11}C]-HVA and [^{11}C]-Me-DOPA whereas the radioactivity in the L-[β-^{11}C]DOPA [^{11}C]-DA and [^{11}C]-DOPAC fractions were considerably lower (Table 1). The yield through the method including homogenization, filtration of the supernatant and liquid chromatographic procedure was 82.2 ± 10.3% and 78.6 ± 15.3% for the striatum and cerebellum, respectively.

Microdialysis samples. The total radioactivity in the dialysate increased with time (Fig. 3). Forty minutes after administration of L-[β-^{11}C]DOPA, 29 and 23%, of the radioactivity were found in the [^{11}C]-HVA and [^{11}C]-DOPAC fractions, respectively. Radioactivities in the [^{11}C]-DA and L-[β-^{11}C]DOPA fractions constituted 9 and 28%, respectively, while [^{11}C]-Me-DOPA constituted about 12% of the total radioactivity in the microdialysate collected at 40 min (Fig. 3).

Synthesis rate of dopamine in the striatum

The synthesis rate of DA in the striatum was calculated to be 0.018 min^{-1} based on the analysis of [^{11}C]-DA and its radioactive metabolites (Table 2). A rate constant of 0.015 min^{-1} was obtained, when the calculation was based on a method similar to the method employed in the PET studies, i.e. the ratio of radioactivity in the striatum to the cerebellum (Table 2).

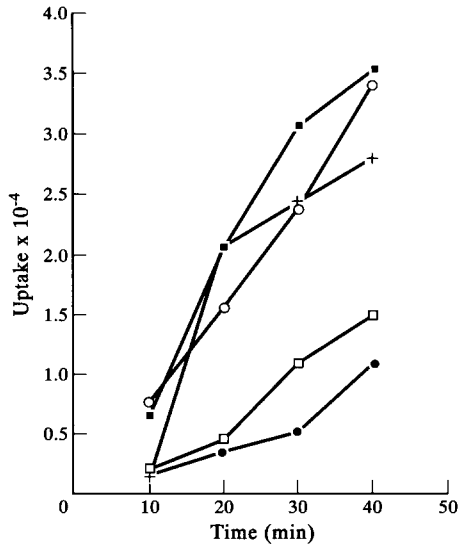

Figure 3
Time course of normalized radioactivity uptake in microdialysate obtained from the rat striatum associated with L-[β-^{11}C]DOPA (○), [^{11}C]-DOPAC (+), [^{11}C]-HVA (■), [^{11}C]-DA (●) and [^{11}C]-3-*O*-methyl-DOPA (□). The microdialysate was analysed using HPLC and the uptake was calculated as described in the text.

Discussion

The accuracy of neurotransmitter synthesis rate obtained *in vivo*, indirectly measured using PET, required a validation including analysis of the composition of the radio-signal from the tissue. In this study, liquid chromatographic determination of intra- and extracellular concentrations of L-[β-^{11}C]DOPA and its metabolites was used to verify rate constants obtained by PET. With the aim of reducing total analysis time, two different parallel chromatographic systems were used for the

Table 2
Dopamine synthesis rate evaluated with different methods

Technique	Species	Calculation	Rate (min^{-1})
Tissue-radioactivity	Rat	Striatum/cerebellum radioactivity ratio	0.015
Homogenate	Rat	Striatum*	0.018
PET [3]	Monkey	Patlak analysis	0.012–0.015
PET [4]	Human	Patlak analysis	0.013–0.016

*Formation rate of [^{11}C]-DA and derived radioactive metabolites in the striatum.

separation of L-[β-^{11}C]DOPA and *in vivo* formed metabolites derived from microdialysis and brain homogenate. As mentioned earlier, one chromatographic system with electrochemical detection was used for the analysis of the composition in the microdialysates, whereas a UV detector was employed when analysing homogenates. The latter system also included an automated fraction collection. However, electrochemical detection could also have been applied for the analysis of homogenates, but since this system was occupied for the analysis of the microdialysates an additional chromatographic system was used for the separation of L-[β-^{11}C]DOPA and radiolabelled metabolites. Separation conditions relied on rapid procedures with adequate sensitivity for radiometric detection. The endogenous levels of L-DOPA and 3-*O*-methyl-L-DOPA were too low to detect in the microdialysate, also reported by others [12]. On the other hand, endogenous levels of DA, DOPAC and HVA were detected (Fig. 1). A standard solution was injected before and after analysis of the microdialysates to observe if the retention times of the compounds remained unchanged during the analysis. Complete resolution of the components required about 10 min.

For the analysis of L-[β-^{11}C]DOPA and radiolabelled metabolites from homogenate, complete resolution of the components required some 14 min. The endogenous levels of L-DOPA and metabolites were too low to detect using UV-detection. Therefore, a standard solution was added to the supernatant to secure that variations in retention could be observed. A simple isolation by precipitation preceded the analysis of brain homogenate samples, whereas microdialysates were injected directly into the chromatographic system. Within the time available for analysis of 1.5–2 h, including sample work up and measurement of radioactivity, analysis of four microdialysates and two brain homogenate samples were performed.

The dopamine synthesis rate in rat striatum was calculated using the information of radioactive composition in the tissue. Provided linear kinetics and that the [^{11}C]-HVA and [^{11}C]-DOPAC found was formed from neuronal [^{11}C]-DA in the dopaminergic terminals in the striatum, the synthesis rate of [^{11}C]-DA was estimated. The results obtained from the rat enabled a confirmation that the rate calculated by PET in humans [1] and monkeys [3] were similar to the *in vivo* synthesis rate of the transmitter [^{11}C]-DA from L-[β-^{11}C]DOPA (Table 1). Although differences in decarboxylase activity between species may exist, the close agreement of the rates calculated either from formation of DA measured in brain tissue or employment of the mathematical models used with PET indicate a similar measure of dopamine synthesis rates (Table 2). In the striatum and cerebellum equal uptake values of [^{11}C]-Me-DOPA were found (Table 1), presumably formed in peripheral tissues, indicating a uniform distribution in the brain.

The advantage of using tissue analysis together with brain microdialysis was a simultaneous assessment of both *in vivo* synthesis as well as release of neurotransmitter to the extracellular space, confirming the compartmentalization of found [^{11}C]-DA intracellularly. Only a small amount of [^{11}C]-DA was found in the dialysate, in agreement with previous reports [7, 8]. This may be accounted for dopaminergic nerve terminal storage as well as the effective inactivation mechanisms of dopamine, e.g. re-uptake and metabolism. However, increasing amounts of the acidic [^{11}C]-DA metabolites, [^{11}C]-HVA and [^{11}C]-DOPAC, were found during the time of the experiment indicating a slow removal of the metabolites from the neuronal extracellular space (Fig. 3).

Thus, the limited specificity of the PET can be overcome by careful validation of the procedure used for quantitation of physiological processes. The validation requires complementary techniques including chromatographic analysis of the composition of the radiosignal.

Acknowledgement — Financial support by the Swedish Medical Research Counci, grant no. 08645, is gratefully acknowledged.

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[Received for review 12 October 1994; revised manuscript received 2 November 1994]