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Combination of positron emission tomography with liquid chromatography in neuropharmacologic research

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SUMMARY

Positron emission tomography (PET) is an *in vivo* autoradiographic technique that determines the radioactive distribution and kinetics of a radiolabelled tracer in a tissue. By choice of tracer, it is possible to study physiological processes in living animals and man non-invasively. PET has certain disadvantages such as limited spatial resolution and similtaneous measurement of radiolabelled tracer with the metabolites formed. For an adequate interpretation of the data obtained, complementary techniques such as column liquid chromatography of radioactive composition in blood, plasma, urine and tissue samples have to be used. The prerequisites for any chromatographic technique used for the radioanalysis of substances are speed, high selectivity and high separation efficiency.

Examples from PET studies in combination with chromatographic analysis will be given. The utilitzation of L-dopa in the brain constitutes several steps. Analysis by column liquid chromatography of metabolites in plasma and in monkey brain tissue will make it possible to elucidate different utilization processes of the tracer. Kinetic studies of ^{11}C-labelled neuropeptides such as methionine-enkephalin and substance P revealed high radioactivities in the brain of monkeys. However, simultaneous determination plasma and urine radioactivities using liquid chromatography with radiochemical and photometric detection both indicated that the brain radioactivities emanated to a large extent from ^{11}C-labelled metabolites formed *in vivo*.

Studies with PET using radiotracers having a rapid and extensive metabolism require complementary techniques in the evaluation. High detection selectivity, by combination of photometric and radiochemical detection and rapid and efficient separation, will make liquid chromatography a most important complement in the analysis.

INTRODUCTION

Positron emission tomography (PET) is a non-invasive tracer technique that

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measures the kinetics of a radiolabelled endogenous or exogenous compound in a biochemical process in the tissue of a living animal or man. The technique has found widespread use in clinical oncology for the delineation and classification of tumours and for the evaluation of treatment effects[1]. Measurement of blood flow in the heart and brain and of brain pH are other important clinical applications[2]. Energy metabolism is studied with various tracers in the heart and brain[3]. However, most applications of PET are still in a state of development, and have not been used on a clinical basis.

Studies with PET on receptor binding characteristics have led to great interest in a number of neurological and psychiatric diseases, and studies on the turnover of substrates for transmitter synthesis have been performed. Further, transport of radiolabelled amino acids over the placenta and the transport of radiolabelled drugs in the spinal canal are other applications that have been addressed using PET. In short, PET may visualize any biological event in the body if the choice of radiolabelled tracer and the time perspective are adequate.

The most serious drawback of PET, in addition to limited resolution, is, as in any other radiometric technique, that the detectors will measure any signal irrespective of its emanation from the radiotracer itself or from metabolites formed during the course of the study. This drawback is particularly troublesome in studies of processes that rapidly yield metabolites in high concentrations. Such interferences can be detected only by simultaneous assay of radiolabelled metabolites with the radiotracer in different body fluids by means of rapid and efficient chromatographic techniques. This paper reviews several applications of chromatographic assays combined with PET, the results of which have been essential in understanding the process under study.

PRINCIPLES OF POSITRON EMISSION TOMOGRAPHY

PET involves three main procedures (Table I), as follows.

TABLE I

POSITRON EMISSION TOMOGRAPHY

Activity	*Location*
Radionuclide production	Tandem accelerator
Radiopharmaceutical synthesis	Organic chemical laboratory
Patient investigation	Positron emission tomography

Radionuclide production

The first step is the production of the radionuclide of choice at the Tandem Accelerator Laboratory at the University of Uppsala. Nitrogen gas is bombarded by a proton beam to form ^{11}C. Trace amounts of oxygen in the target gas will yield $^{11}CO_2$.

Synthesis and analysis

The second step is the conversion of $^{11}CO_2$ in a series of steps to a useful ^{11}C-labelled precursor. In many cases, [^{11}C]methyl iodide has been used, which is a valuable alkylating reagent. For example, the corresponding demethylated analogues are reacted with [^{11}C]methyl iodide, using spiperone in an N-alkylation to give N-[^{11}C]methylspiperone or using S-benzylhomocysteine in an S-alkylation to give [^{11}C]methionine. After separation of the product by semipreparative liquid chromatography[3], the preparation and control of the product with respect to chemical and radiochemical purity are performed by analytical liquid chromatography. The half-life of ^{11}C is 20.4 min, which means that only a limited time period is available for production of the radiotracer by rapid organic chemical procedures and for purification. Additional requirements of the tracer administered to the patient are a sterile and pyrogen-free preparation.

Tomography

The third step is the investigation of the patient. The patient lies in a fixed position in the positron emission tomograph. Images from the tomograph are obtained in the following manner (Fig. 1). The radionuclides emit positrons, which, after traversing the tissue for a few millimetres, collide with its antimatter equivalent, the electron. In the annihilation reaction, two antiparallel photons are emitted. The photons are measured by external detectors, and the detector signals are processed and computed to give an exact localization of the annihilation. By measuring sequential images, the change in radioactivity with time in an organ or part of organ can be studied, in addition to its spatial localization. A radioactive uptake, corrected for physical decay of the radiotracer and the radioactive dose per gram of body weight, will characterize the distribution and kinetics of the radiotracer in the region of interest. Using radiolabelled receptor ligands, the difference in radioactivity over time in the receptor-rich region compared with a reference tissue devoid of specific receptors, *e.g.*, cerebellum for dopamine receptor studies, will give information on the number and binding characteristics of the receptor ligand after fitting a model to the data.

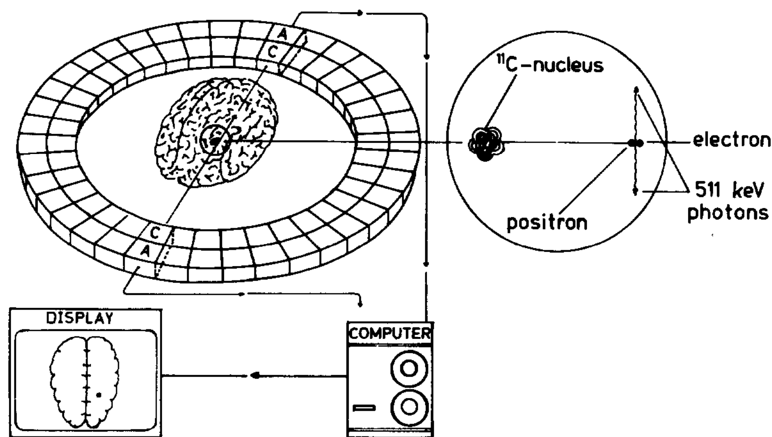

Fig. 1. Principles of positron emission tomography with ^{11}C-labelled tracers.

PHARMACEUTICAL CONTROL

Before administration to man, the radiotracer must fulfill the requirements of a pharmaceutical product to be administered parenterally. The preparation of the product should be achieved in the shortest possible time because of the rapid decay of the radiotracer. Identity, chemical and radiochemical yield, together with sterility and absence of endotoxins, must be ensured in a short time period[3]. In these instances, liquid chromatography is an invaluable tool. A rapid and efficient chromatographic separation, together with the detection selectivity obtained by the combination of radiochemical and photometric detection, are necessary for control of purity and coelution with unlabelled reference compound. The chromatographic analyses are performed using standard conditions frequently employing reversed-phase systems (Fig. 2).

The product is controlled by two independent analyses using liquid chromato-

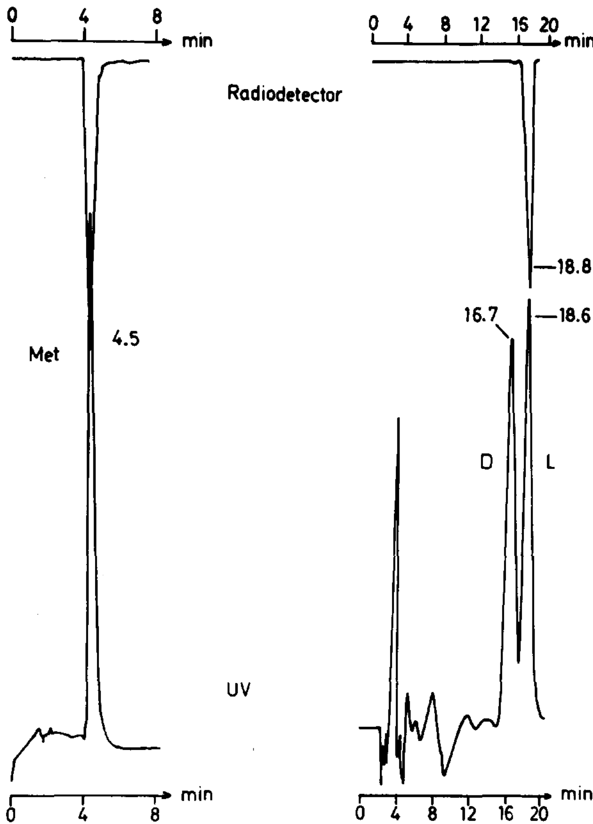

Fig. 2. Qualitative liquid chromatographic determination of [^{11}C]methionine used in clinical oncology for the delineation and classification of tumours and the evaluation of tumour treatment. A photometric detector is used in series with a β-detector as radiodetector. Left: radiochemical analysis on reversed-phase C_8-amino column. Amount injected, 5 MBq of [^{11}C]methionine corresponding to about 0.5 ng and standard addition of methionine. Right: analysis of the optical enantiomers of [^{11}C]methionine on an enantiomeric column packing (Daicel N) after standard addition of unlabelled reference.

graphy, for example one on the preparative scale and one on the analytical scale. Addition of the corresponding reference compound to the radiolabelled compounds and then reanalysis by liquid chromatography will further strengthen the control. Monitoring of the eluate with both radiochemical and photometric detectors will give both the chemical and radiochemical purity of the product, respectively. After dissolving the product in the appropriate buffer, the preparation is thereafter filtered through a 0.22-μm filter in a laminar flow hood to give a sterile product. The control tests are approved by the hospital isotope committee before patient studies. The total time for pharmaceutical preparation does not take more than 10 min.

APPLICATIONS

PET studies of receptor agonists and antagonists

Studies on the alterations of specific receptors in disease are field of great interest in using PET. Symptoms of many psychiatric and neurological disease are successfully relieved by treatment with receptor-active drugs. Any indication of an alteration of receptor binding may be of importance for a further understanding of the etiology. Blockade of dopamine D_2 receptors is the main pharmacological action of antipsychotic drugs. Although still debated, there are now several independent reports of unchanged dopamine D_2 receptor binding in schizophrenic patients. Furthermore, no changes in dopamine D_2 receptor binding have been shown with PET in other disorders such as tardive dyskinesia and in more advanced stages of Parkinson's disease. Although studies on dopamine D_2 receptor binding has been largely negative, other important aspects of specific receptor binding can be addressed with PET.

Clozapine is an atypical antipsychotic drug with a superior clinical effect coupled with a low incidence of extrapyramidal side-effects and the absence of induced tardive dyskinesia. On the other hand, rapidly after termination of clozapine therapy, psychotic symptoms which may have serious consequences may return. PET studies in monkey[4] and man[5] revealed an accumulation of brain radioactivity to the striatal region and cortical areas. Interaction with pharmacological doses of a selective D_2 receptor antagonist, haloperidol, showed part of the striatal binding to be at selective dopamine D_2 receptors. Binding to the serotonin (5HT-2) receptor in the frontal cortex of Rhesus monkey was suggested[4]. Binding to other receptor populations must also be accounted for. The binding of clozapine to several receptor populations, as indicated with PET, might be of significance in the good clinical effect seen in schizophrenic patients[5].

Quantification of the receptor binding characteristics of [^{11}C]clozapine revealed a high dissociation rate from the receptors. In fact, the rate of elimination of radioactivity from the brain was similar to the plasma elimination rate of intact clozapine[4]. Simultaneously with the radioactive dose, a pharmacological dose of unlabelled clozapine was given to the monkey, and blood samples were withdrawn at regular intervals after dosing. After extraction into an organic phase and further purification, clozapine was determined by gas chromatography–mass spectrometry with selected ion monitoring[4]. A loose receptor binding for clozapine may have implications both for the low incidence of extrapyramidal side-effects and the occurrence of psychotic symptoms early after drug withdrawal.

Brain L-dopa utilization studied with PET

Dopaminergic nerve function has been addressed in many PET studies owing to the cumulation of dopamine nerve terminals in the striatum-substansia nigra area in the brain, and to the relative availability of tracers that will selectively measure the processes within the synaptic cleft. [^{18}F]Fluoro-dopa utilization in the brain has been studied both in health and disease for several years. The [^{18}F]fluoro-dopa-derived radioactivity represents a complex system, and several processes in dopamine turnover are visualized. After systemic administration of [^{18}F]-L-dopa, the radioactivity is transferred rapidly over the blood–brain barrier, taken up into the dopaminergic terminals, converted by decarboxylases to dopamine (which is stored in the vesicles) and released eventually to the postsynaptic receptors or metabolized. Metabolic enzymes such as monoamine oxidase and catecholamine-O-methyltransferase yield a complex pattern of radioactive metabolites[6].

Hence, a defective L-dopa brain turnover is difficult to visualize with PET, and several complementary methods have to be used. Recently, Firnau *et al.* used column liquid chromatography to separate the different F-labelled metabolites in both plasma[7] and brain tissue of monkey[6]. Plasma and brain tissue samples were analysed by reversed-phase liquid chromatography. The eluent was collected into fractions, which were measured for radioactivity[6]. The peaks representing ^{18}F radioactivity were identified by comparison of their elution times with those of the authentic ^{18}F-labelled catechol materials chromatographed under identical conditions. A simultaneous detection with both radiochemical and photometric detection would increase the performance of the procedure. From these studies[6,7], it was concluded that about 70% of the radioactivity in the striatum was due to the formation of [^{18}F]dopamine. [^{18}F]-3-O-methyl-dopa constituted a large fraction of the radioactivity in the cortical regions and the cerebellum. This finding is an obstacle for an adequate *in vivo* calculation of dopamine synthesis rate using cerebellum as a reference.

A second way to overcome this problem is to block selectively the metabolic enzymes monoamineoxidase and catecholamine-3-O-methyltransferase one by one in animal experiments[8]; thereby the contribution of each metabolic step to the brain radioactivities can be assessed more adequately.

A third and interesting approach is to label the molecule of interest in different positions[8]. Labelling of L-dopa with ^{11}C in the 1-position in the carboxylic acid group means that the label is lost in the decarboxylation step to dopamine. The $^{11}CO_2$ formed is eliminated from the brain. Hence this tracer gives information on the input rate to the brain and the rate of decarboxylation.

L-Dopa labelled with ^{11}C in the 3-position is transferred to the brain so that the radiolabel is withheld in all metabolic steps. This tracer gives the full information on brain L-dopa utilization.

Only by using a combination of all three approaches can a full understanding of dopamine brain turnover be reached.

Brain kinetics of neuropeptides studied with PET

Many peptides are produced both in the central nervous system and in peripheral tissues. The endogenous agonists for the opioid receptors, the opioid peptides endorphins, are typical examples. There have been discussions as to whether endorphins produced in peripheral tissues cross the blood–brain barrier and induce signifi-

cant central activity. The smallest opioid peptides, the enkephalins, are relatively unstable in plasma, and an extensive metabolism may hamper their transfer to the CNS. Enkephalin analogues that are metabolically stable, on the other hand, are known to be analgesic after systemic administration, indicating passage over the blood–brain barrier.

These issues have also been studied using PET in combination with column liquid chromatography[9]. The enkephalins studied were methionine-enkephalin and three synthetic analogues, Tyr–D-Ala–Gly–Phe–Met–NH_2 (DALA), Tyr–D-Met–Gly–Phe–Pro–NH_2 and Tyr–D-Ala–Ala–Phe–Met–NH_2. They were all labelled with ^{11}C in the methyl group of methionine. The kinetics of the radioactive distribution to the Rhesus monkey brain was visualized with PET and the elimination in Rhesus monkey plasma *in vivo* and *in vitro* were measured by reversed-phase liquid chromatography. At regular intervals, blood samples were collected and the plasma was separated. The plasma was filtered through a Sephadex G-25 PD-10 column. A sample from the low-molecular-weight fraction was injected onto a Spherisorb C_{18} column with a variable-wavelength photometric detector and a β-flow detector. Gradient LVC programmes were carried out using 0.1 *M* aqueous ammonium formate (pH 3.5) and methanol as the mobile phase. Fractions of the eluate were collected, and the radioactivity was measured in *in vivo* studies. Photometric detection of the eluent for the column had sufficient sensitivity for *in vitro* analysis. A chromatogram is shown in Fig. 3.

Analysis with PET showed an increased radioactivity in the monkey brain over the 60–90-min investigation period following intravenous administration of the enkephalin peptides. Highest brain radioactivities were measured for injections of methionine-enkephalin and DALA. However, a large fraction of brain radioactivity derived from these two enkephalin peptides probably emanated from ^{11}C metabolites formed *in vivo*, as evidenced by chromatographic analysis of plasma and urine samples from the monkeys. Methionine-enkephalin was cleared rapidly from plasma *in vitro* with a half-life of less than 2 min, whereas DALA was found to be stable *in vitro*. ^{11}C radioactivity in the brain originated from ^{11}C metabolites formed from methionine-enkephalin and DALA. For the two other enkephalin peptides, only minor concentrations of plasma ^{11}C metabolites were determined.

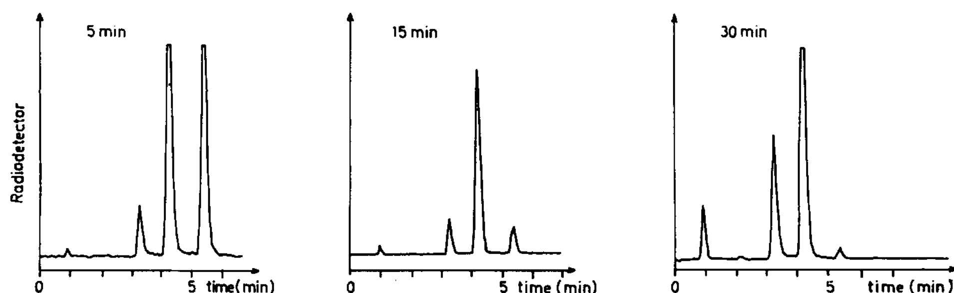

Fig. 3. Analysis of the *in vitro* metabolism of [^{11}C]methionine-enkephalin in plasma using a reversed-phase Spherisorb C_{18} column equipped with a variable-wavelength UV detector, showing the rapid hydrolysis of [^{11}C]methionine-enkephalin to polar ^{11}C-labelled metabolites.

Substance P labelled with ^{11}C in the methyl group of methionine showed similar results with high radioactivities in brain. After analysis of plasma samples by liquid chromatography, high concentrations of ^{11}C metabolites were seen. Hence, in the case of ^{11}C-labelled substance P, the brain radioactivities probably also emanated from radiolabelled metabolites formed.

These conclusions concerning the brain distribution and kinetics of ^{11}C-radiolabelled neuropeptides could only be reached by a careful combination of PET with studies using liquid chromatography on the *in vivo* and *in vitro* metabolism of the peptide.

CONCLUSIONS

The new imaging technique PET visualizes specific biochemical processes in the tissues of a living animal and man, and the information given cannot be obtained with any other method. The results and conclusions obtained with PET have been achieved only by combination with other measurement techniques. The most important and powerful of these other methods is the liquid chromatographic analysis of the radioactive constitution of different body fluids and tissues. Liquid chromatography also plays an important role in the purity control of the radiolabelled product before administration to a patient. The high detection selectivity achieved by the simultaneous use of radiochemical and photometric detection had to be combined with a rapid and versatile chromatographic separation with good selectivity and high separation efficiency.

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