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Development of Robotic Plasma Radiochemical Assays for Positron Emission Tomography

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ABSTRACT

A commercial laboratory robot system (Zymate PyTechnology II Laboratory Automation System; Zymark Corporation, Hopkinton, MA) was interfaced to standard and custom laboratory equipment and programmed to perform rapid radiochemical analyses for quantitative PET studies. A Zymark XP robot arm was used to carry out the determination of unchanged (parent) radiotracer in plasma using only solid phase extraction methods. Robotic throughput for the assay of parent radiotracer in plasma is 4-6 samples / hour depending on the radiotracer. Robotic assays of parent compound in plasma were validated for the radiotracers [^{11}C]Benztropine, [^{11}C]cocaine, [^{11}C]clorgyline, [^{11}C]deprenyl, [^{11}C]methadone, [^{11}C]methylphenidate, [^{11}C]raclopride, and [^{11}C]SR46349B. A simple robot-assisted methods development strategy has been implemented to facilitate the automation of plasma assays of new radiotracers.

INTRODUCTION

Positron Emission Tomography (PET) is a nuclear medicine imaging modality that allows the quantitative observation of the time course of spatial distributions of positron-emitting radiopharmaceuticals in the organs (typically brain and heart) of awake human subjects after intravenous injection. Combined with plasma radioactivity measurements and a validated mathematical model, this *in vivo* radioactivity distribution data can be used to characterize specific biochemical systems (e.g. neurotransmitter receptor systems) important to normal organ and tissue function. Often, multiple plasma radiochemical assays - including a series of labeled metabolite correction determinations - must be made for every quantitative PET procedure, requiring additional personnel working in a potential hazardous environment. This metabolite-corrected, plasma time course of radioactivity is often referred to as the *input function* for the study.

Automated devices for determination of input functions would reduce both personnel costs and health risks associated with handling human blood at PET facilities. Plasma input function determination involves two different radiochemical assays: (1) the determination of the total *plasma* radioactivity concentration, and (2) the determination of the *fraction of unchanged radiotracer* contributing to the total *plasma* radioactivity (called the parent compound assay). We have recently described in detail our robotic system for carrying out such assays (1). A uniqueness and power of the PET method, however, is the potential use of dozens of *new* radiotracers being developed in our laboratory and others every few years. Therefore, the ability to develop quickly new parent compound assays is an important goal of our automation efforts which support both in-house baboon and human studies. This article describes the methodological constraints and goals of the robotic plasma assay and how such methods are developed and validated in our laboratory.

MATERIALS AND METHODS

Laboratory Automation Hardware

The complete description of the laboratory hardware comprising our robotic input function determination system is described in (1). The hardware listed below describes the components needed to develop new, and run existing, solid phase extraction assays for input function determination. The laboratory automation system (Zymark PyTechnology II) consisted of the following PySection™ compatible hardware/software modules from Zymark Corporation, Hopkinton, Massachusetts: XP robot arm, a general purpose hand for manipulating 16 x 100 mm glass test tubes (ZP900-1), two 50-position 16 x 100 mm test tube racks (ZP013-3), a six solvent Liquid/Solid Extraction PySection™ (ZP731-1), a Dilute and Dissolve (w/vortex) PySection™ (ZP620-2) used for filling sample tubes, three Master Laboratory syringe pump PySections™ (ZP510), and a Power and Event Controller input/output PySection™ (ZP830).

The solid phase extraction station was modified to include liquid level sensing using a commercial optical proximity detector. The SmartEye™ (Tritronics, Tampa. FL) detector is mounted to the station just under the pneumatic nozzle so that light emitted from the detector is reflected back by the presence of an extraction cartridge. Slotted brackets permit limited vertical , depth, and horizontal adjustment. Both the power (±12VDC) for the detector and the analog output (12VDC f.s.) are connected directly to the robot's Power and Event Controller. The detector's output signal is used to verify the presence of a cartridge and to control the duration of solvent elution on the cartridge. Zymark PySection software was modified so that the change in detector voltage accompanying a transition from wet to dry is compared to a calibrated "done" threshold automatically during program execution.

A custom "PySection" for radioactivity measurement using a NaI (3 inch crystal) well counter was constructed and interfaced with Zymark's PyTechnology system. The custom PySection accepts standard 16 x 100 mm test tubes and provides a two-position lead rack for temporary sample storage. A Nylon insert for the well counter was built to locate reproducibly either a 16 x 100 mm test tube or a 500 mg BondElut LRC (Varian, Harbor City, CA) SPE cartridges in the center of the well. An extraction cartridge parking station made from a no. 7 rubber stopper, mounted upside

down, also resides on the lead rack. The robot turns a standard extraction cartridge upside down and rests it on the inverted stopper attached to the lead rack. The stopper ensures that the cartridge remains upright while the robot positions itself to regrip the cartridge at the standard container grip position before inserting it into the well counter.

A custom hazardous waste disposal "PySection" was also constructed for the robot's table. The hazardous waste station consists of a three-tiered disposal system designed for recycling and sorting solid waste. Empty cartridges used for purging the solvent delivery lines to the solid phase extraction station are recycled by the robot. Plasma-contaminated cartridges and test tubes are sorted and placed directly into appropriate waste containers (sharps for human waste) residing directly on the robot table. Liquid waste is accumulated in two 5 gallon polyethylene containers residing on the floor next to the robot table. The infectious waste container is pre-filled with bleach to give a 10% final concentration when full. This precaution is needed for sterilizing before disposal. The other drum accumulates non-infectious waste only.

The laboratory automation system is controlled by Zymark's custom microcomputer and operating system named System V (ver. 1.52) running System Productivity Software (SPS) version 1.3. This system is controlled remotely from an IBM PS/2 Model 60 microcomputer running DOS 3.30 using Zymark's high speed serial interface and PCREMOTE software. The PS/2 is also connected via RS-232 to standard NIM electronics interfacing the NaI well counter. The signal output from the counter/timer is connected to one of the digital inputs (TTL) at the Power and Event controller. This signal allows the robot to determine when the counter has finished counting a sample.

Software

Robot applications software was developed at BNL using Zymark's Easylab™ laboratory automation control language. These Easylab™ programs control all container movement and liquid handling. Custom "Py-compatible" software was developed to support radioactivity counting of 16 x 100 mm tubes and BondElut cartridges, and for hazardous waste disposal. Easylab software supporting all Zymark PySections was commercially available from Zymark, including 16 x 100 mm test tube "pouring" subroutines. IBM BASICA programs on the PS/2, executed interpretively, synchronize robot, counter, and operator

functions while acquiring radioactivity data directly from the counter. Figure 1 illustrates this relationship of Zymark's System V controller and the IBM PS/2. Three EasyLab(TM) interpreters running concurrently on the System V controller provide simultaneous control of the XP arm, liquid handling at the solid-phase extraction station, and bi-directional, serial communication with the PS/2.

During normal operation sample input from the operator is synchronized through the serial communication port using the tactile sensing of the robot and the keyboard of the PS/2 in the following manner. The error recovery routine of the EasyLab program GET.FROM.RACK.1 was modified so that a "warm start" procedure was initiated when the proper command was received from the remote link. This was necessary since samples are delivered to the robot's input rack from a different building and are spaced sparsely (up to 30 min.) in time.

Parent compound Analysis by Robotic SPE

Baboon (*Papio anubus)* or human plasma samples (0.05 mL to 1.0 mL) - obtained manually from whole blood by a PET clinical laboratory worker - are added to robot test tubes containing 3 mL of deionized water. The samples are stored on ice until placed in the robot's input test tube rack after which time the sample tube is counted 1 minute for annihilation photons (511 keV) in the Py-compatible NaI well-counter. During radioactivity counting, a solid-phase extraction cartridge is retrieved and rinsed with solvent. While this cartridge is conditioning, the arm returns to the NaI counter station to retrieve the sample tube and pour its contents onto the wetted column. During the pour, a background radioactivity measurement is started. The empty sample tube is then counted and returned to a rack while the liquid in the extraction cartridge is pushed slowly into an awaiting clean and dry test tube. This fraction, named the dead volume, is counted while another solvent rinse of the cartridge is collected into a new test tube. The cartridge is rinsed with several solvents to remove the more polar metabolites and each fraction is counted and returned to a test tube rack for storage. Finally, the robot arm retrieves the SPE cartridge, assays its radioactivity, and disposes it at the hazardous waste station.

This simultaneous sequence of events including shuffling tubes and cartridges, acquiring radioactivity data, and moving liquids through the cartridge continues until all the samples are processed. Typically eight samples are processed for each study using a sampling protocol of 1,5,10,20,30,45,60,90 minutes post injection.

Validation of Robotic Methods

Validation of all robotic SPE-only assays is comprised of three steps:

1. *Characterization of plasma loading*
2. *Correlation with independent manual HPLC assay*
3. *HPLC analysis of "pure" SPE fraction at late (>30 min.) times*

The effect of plasma volume on the recovery of the robotic SPE method was determined by adding plasma (0.05 mL to 1.0 mL) to a standard robot test tube containing 3 mL of deionized water spiked with 30,000 to 300,000 counts per minute radioactivity as pure radiotracer (e.g. [^{11}C]methadone). These samples were processed by the robot using a SPE method (see above) under validation.

Detailed conditions for HPLC analyses are described in (1). Each robotic SPE method is compared to HPLC analyses carried out by chemists, and the radioactivity defined as "pure" radiotracer is also subject to HPLC analysis to confirm that this "pure" radioactivity fraction is in fact due mostly to the presence of the radiotracer and not any metabolites.

Data Analysis

The quantity of parent compound present in each plasma sample is reported as a percent of the total radioactivity based on the radioactivity measured in the "pure" fraction - usually the SPE cartridge - after correction for background radioactivity, container geometry-dependent counting efficiency (1), total radioactivity recovery, and radionuclide decay. Template worksheets in Lotus 1-2-3™ for each radiotracer contain all the formulas required for data reduction. Data is

saved automatically by the BASIC program and imported directly into the tracer-specific worksheets.

RESULTS and DISCUSSION

The most common method for the determination of the fraction of radioactivity in plasma present as unchanged radiotracer uses High Performance Liquid Chromatography (HPLC) following the removal of plasma proteins by precipitation or solid-phase extraction (2). Such HPLC methods must incorporate standard effluent radioactivity counting equipment. Since the half-lives of radionuclides used in PET are very short (20.4 minutes for ^{11}C), these methods must be both fast and efficient. To make metabolite corrections at longer time-points in a study, often > 60 minutes after injection, larger plasma samples must be used to compensate for the lower radioactivity count rates present due to the physical decay of the radionuclide. Unfortunately, standard HPLC methods used in PET plasma analyses are often limited in the volume of plasma that can be processed for each sample. In part to compensate for these potential limitations, a strategy evolved in our laboratory around the strengths of our laboratory robot in automating sample preparation steps leading to the development of rapid automated parent compound assays using only solid-phase extraction methods.

As new radiotracers are introduced into the field and our laboratory, new assays are validated for the robot SPE-only system. All of these methods are developed with the following goals in mind:

1. *No precipitation of proteins*
2. *> 90% selectivity*
3. *> 90% retention of radiotracer*
4. *> 90% recovery of compound for HPLC validation*

The first goal simplifies the assay procedure to maximize sample throughput. This is essential for working with short-lived positron-emitting radionuclides like carbon-11. The remaining specifications are a reflection of practical constraints on radiation counting and the accuracy required in such measurements for PET studies. PET data is often analyzed in a manner where only changes from the first study to the second, with the subject being his/her own control, are reported. Selectivity is defined as the relative

amount of parent radiotracer present in the "pure" fraction of the assay. This fraction is most often the SPE cartridge. Retention is defined as the relative amount of parent tracer retained on the SPE cartridge as determined by plasma loading experiments. The last goal only relates to the final validation of a method.

Most often the problem of separating all metabolites from the parent compound in a PET study can be reduced to the problem of separating more polar molecules from their larger more nonpolar parent radiotracer. Only the radioactive metabolites need be separated, further simplifying the assay. Figure 2 shows the simple methods development process used in our laboratory to develop robotic SPE-only assays of PET radiotracers in plasma to meet these goals. To illustrate the evolution of this robot-assisted methods development, all of the figures appear using data from the compound [^{11}C]raclopride, a common PET radiotracer used to assess dopamine receptor availability in human (3).

Results of a screening protocol that included a basic aqueous wash and several washes of varying methanol/water mixtures is presented in Figure 3. This figure shows a clear shifting of the [^{11}C]raclopride radioactivity from the mixtures of less methanol to more methanol as the polarity of the bonded-phase is decreased. To illustrate clearer this difference, Fig 4 shows two of these bonded phases. Following the simple logic depicted in Fig. 3, these same bonded phases were used in a baboon experiment to determine the selectivity of the bonded phases for [^{11}C]raclopride and its radioactive metabolites at a single time-point post injection. It is clear from the results in Table 1 that the CN bonded-phase was much more selective in its retention for [^{11}C]raclopride than its metabolites. This finding lead to the development of a SPE-only method based on the CN bonded phase an sufficient water washes. This method was then validated by HPLC (see Fig. 5). Note that both the metabolite fraction and the unchanged fraction are highly correlated, which confirms that the fraction of radioactivity defined as [^{11}C]raclopride in the SPE method is mostly (>90%) [^{11}C]raclopride.

Plasma loading experiments showed no significant (< 10%) breakthrough of [^{11}C]raclopride up to 0.4 mL of baboon plasma. This was *not* true for human plasma. The results of human plasma loading results are shown in Fig. 6. This figure also illustrates the LOT number dependence of radiotracer retention. Others have reported significant LOT number effects on SPE (4). Because of this finding, control experiments are done with every new LOT number to assess maximal

loading. Other radiotracers, like [^{11}C] *d-threo*-methlyphenidiate (5) and [^{11}C]methadone (6), show retention > 98% up to 1.0 mL of plasma. This case is the ideal one since larger plasma volumes give better radioactivity counting statistics and allow the measurement of parent compound in plasma at later times (60 -90 minutes) after injection. It is our experience that these very large plasma volumes pose problems to standard HPLC methods used in PET plasma analyses. Table 2 summarizes the list of robotic SPE-only parent compound assays developed on our Zymark robot and implemented as a routine, automated analyses.

CONCLUSIONS

The Zymark system has not only proved to be reliable and useful for routine automation of this type of analysis, but it has also provided our laboratory with a critical increase in productivity needed to develop *new* assays using the simple strategy outlined here. During the development of new PET radiotracers, many people are required to carry out such tasks as radionuclide production, radiotracer synthesis and quality control, biodistribution studies in rodents, and baboon PET imagin studies. Often, several of these tasks must be carried out simultaneously. Since methods development of new plasma SPE-only assays can only be carried out at the same time because of the necessity for the radioactive compound and its metabolites, additional personnel must be assigned to carry out these experiments. This may only be required for several experiments until a method is validated and handed over to the robot for routine analyses. *It is this dual role, of temporary laboratory assistant and permanent automated device, that has proven so useful in our laboratory.*

In summary, the availability of rich variety of robot-compatible bonded phases for SPE, the off-the-shelf functionality of commercial laboratory robots, and the unique characteristics of plasma analyses for quantitative PET procedures have combined to give a reliable, easy-to-use system used routinely in our laboratory for the past 4 years.

ACKNOWLEDGMENT

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6. Unpublished result from our laboratory.

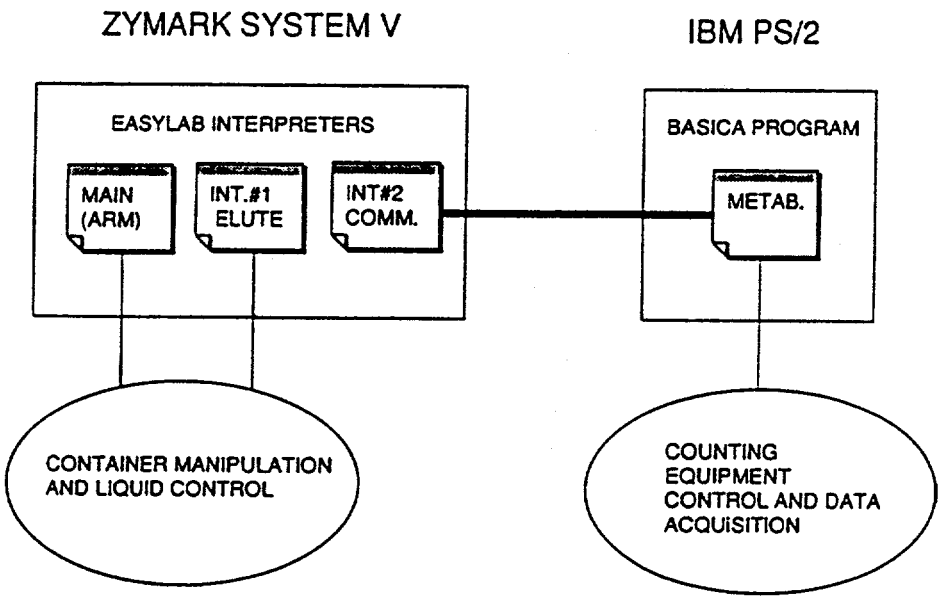


Fig. 1: System configuration of robotic SPE assay system. The PS/2 interfaces the operator and serves as the master computer for the system.

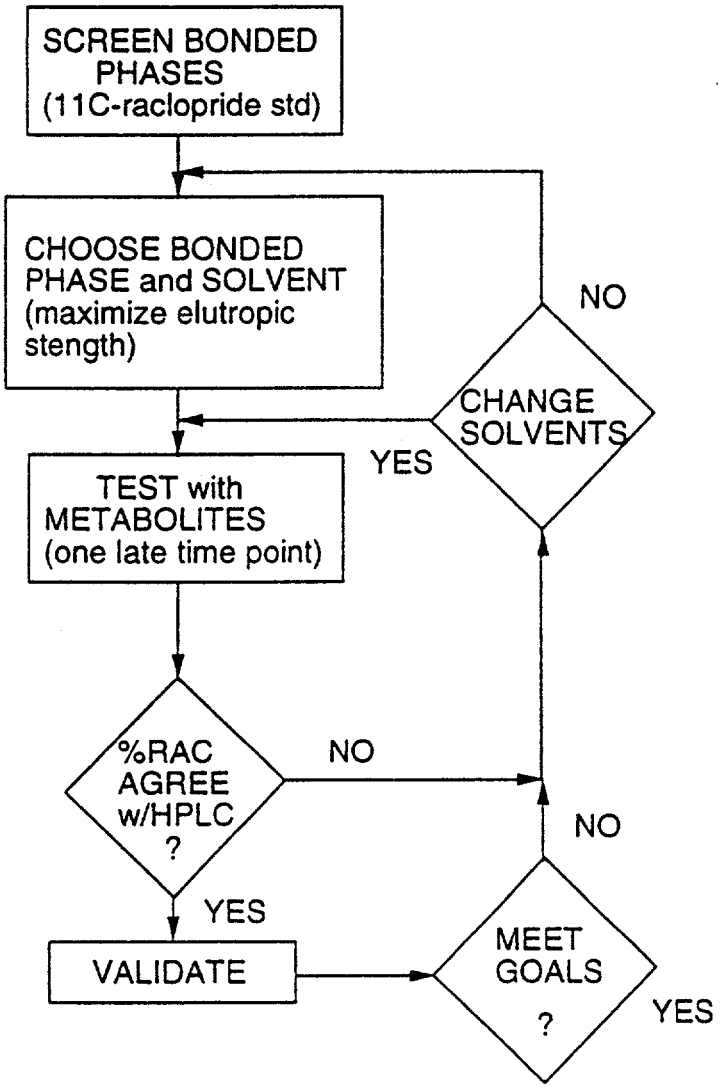


Fig. 2: Simple SPE-only methods development strategy for non-polar extraction of parent compound from plasma.

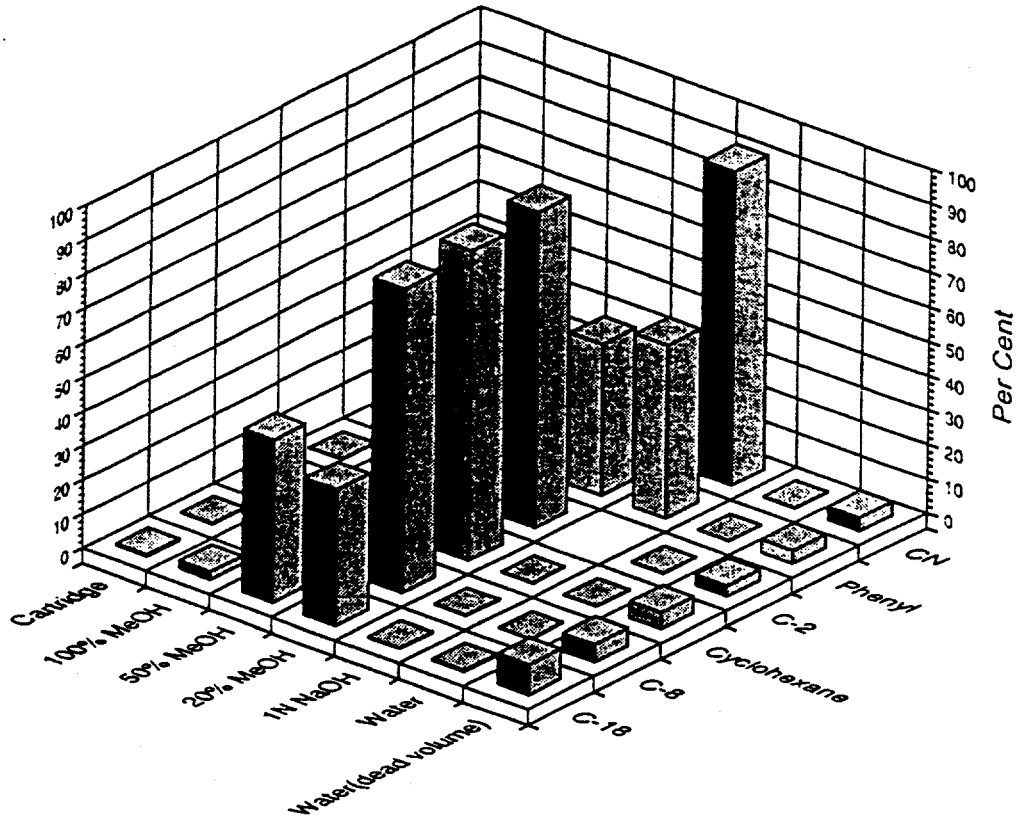
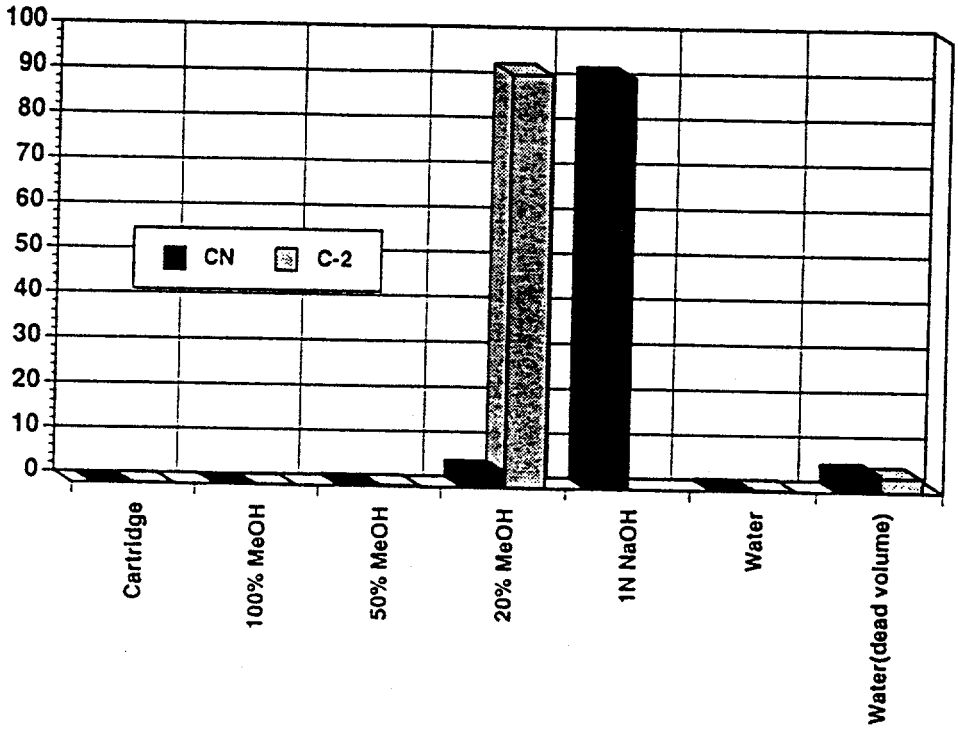


Fig. 3: Results of non-polar extraction screening run with [11C]raclopride

Fig. 4: Extraction of [11C]raclopride from baboon plasma using CN and C2 bonded phases



Method	11C-raclopride at 30 min. PI
HPLC	40%
C2 extraction	76%
CN extraction	47%

Table 1: Comparison of HPLC and SPE determinations of [11C]raclopride in baboon plasma

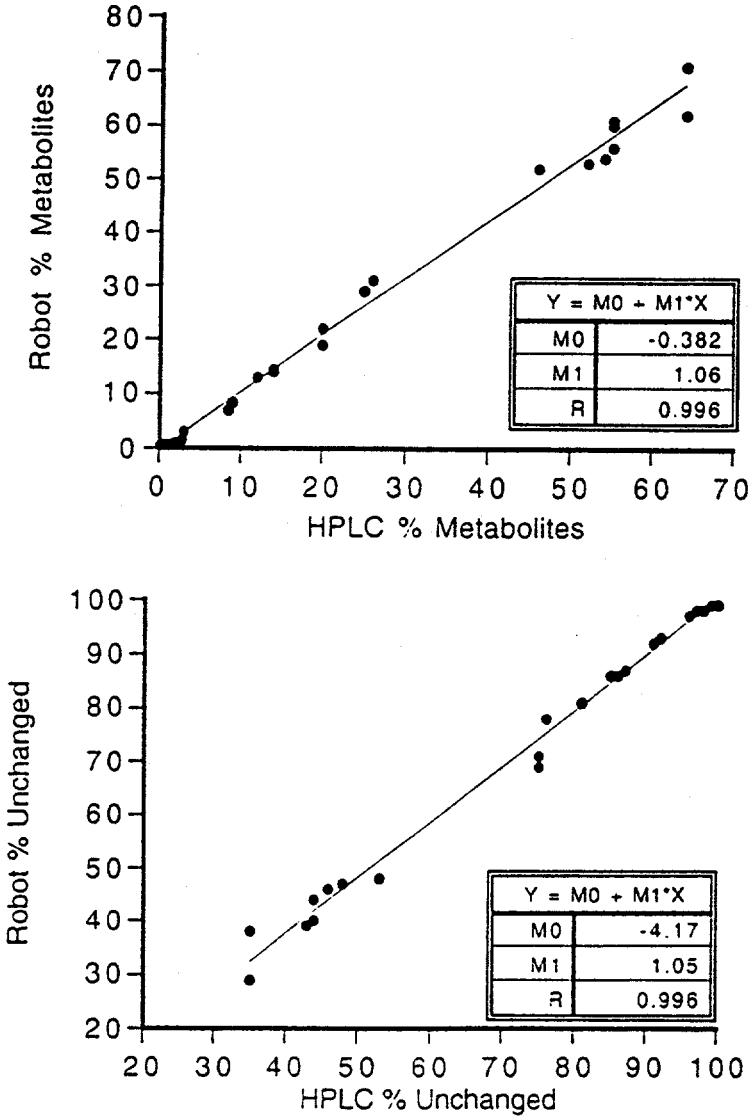


Fig. 5: Comparison of manual HPLC and robotic SPE assays of [11C]raclopride in baboon plasma. Top graph shows correlation of metabolite fractions. Bottom graph compares unchanged fractions

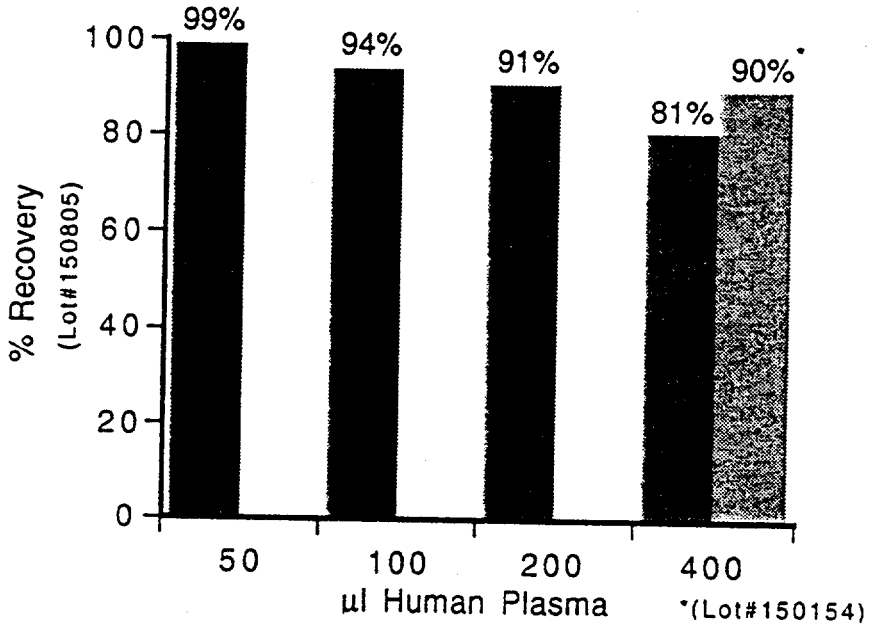


Fig 6: Effect of plasma volume on the recovery of [11C]raclopride

Table 2

Summary of Robotic SPE Parent Compound Assays

Radiotracer	Plasma	SPE	Rinse 1 (mL)	Rinse 2	(mL)	Rinse 3	(mL)	Rinse 4	(mL)	Unchanged*
[^{11}C]Cocaine	hum/bab	C18	water (5)	60% ME	(5)	60% ME	(5)			C18
[^{11}C]Benztropine	hum/bab	CN	water (5)	water	(5)	water	(5)			CN
[^{11}C]Clorgyline	human	CN	water (5)	100% ME	(6)					100% ME
[^{11}C]Deprenyl	human	CN	water (5)	100% ME	(6)					100% ME
[^{11}C]Methamphetamine	baboon	CN	water (5)	100% ME	(6)					CN
[^{11}C]Raclopride	human	CBA	water (5)	pH 7 PB	(5)	pH 7 PB	(5)			CBA
[^{11}C]Raclopride	baboon	CN	water (6)	water	(6)	water	(6)			CN
[^{11}C]Methadone	baboon	CN	water (5)	water	(5)	water	(5)			CN
[^{11}C]Methylphenidate	human	CN	water (5)	50% ME	(5)					CN
[^{11}C]Methylphenidate	baboon	CN	water (5)	water	(5)	50% ME	(5)	50% ME	(5)	CN
[^{11}C]SR 46349B	baboon	C8	water (5)	60% ME	(5)	60% ME	(5)			C8

ME - methanol
PB - phosphate buffer
C8 - non-polar (octyl)
C18 - non-polar (octadecyl)
CN - non-polar (cyanopropyl)
CBA - weak cation exchange (carboxylic acid)