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Rapid Solid-Phase Extraction Method to Quantify [¹¹C]-Verapamil, and its [¹¹C]-Metabolites, in Human and Macaque Plasma

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Abstract

Introduction—P-glycoprotein (P-gp), an efflux transporter, is a significant barrier to drug entry into the brain and the fetus. The Positron Emission Tomography (PET) ligand, [¹¹C]-verapamil, has been used to measure *in vivo* P-gp activity at various tissue-blood barriers of humans and animals. Since verapamil is extensively metabolized *in vivo*, it is important to quantify the extent of verapamil metabolism in order to interpret such P-gp activity. Therefore, we developed a rapid solid phase extraction (SPE) method to separate, and then quantify, verapamil and its radiolabeled metabolites in plasma.

Methods—Using high performance liquid chromatography (HPLC), we established that the major identifiable circulating radioactive metabolite of [¹¹C]-verapamil in plasma of humans and the nonhuman primates, *M. nemestrina*, was [¹¹C]-D-617. Using sequential and differential pH elution on C₈ SPE cartridges, we developed a rapid method to separate [¹¹C]-verapamil and [¹¹C]-D-617. Recovery was measured by spiking the samples with the corresponding non-radioactive compounds and assaying these compounds by HPLC.

Results—Verapamil and D-617 recovery with the SPE method was >90%. When the method was applied to PET studies in humans and nonhuman primates, significant plasma concentration of D-617 and unknown polar metabolite(s) were observed. The SPE and the HPLC methods were not significantly different in the quantification of verapamil and D-617.

Conclusions—The SPE method simultaneously processes multiple samples in less than 5 min. Given the short half-life of [¹¹C], this method provides a valuable tool to rapidly determine the concentration of [¹¹C]-verapamil and its [¹¹C]-metabolites in human and nonhuman primate plasma.

Keywords

P-glycoprotein; PET; SPE; human; *M. nemestrina*; [¹¹C]-verapamil; metabolites; macaque

1. Introduction

P-glycoprotein (P-gp), a 170-kDa membrane ATP-binding cassette efflux transporter, is found in the luminal membrane of endothelial cells that form the blood-brain barrier (BBB), in the syncytiotrophoblasts of the placenta that form the blood-placental barrier (BPB), as well as in the cell membranes of other organs important in drug absorption and disposition [1–3]. At

these sites, P-gp is predominantly responsible for restricting the entry of its substrate drugs or other xenobiotics into tissues (e.g. the brain and the fetus) or for drug absorption and elimination (e.g. the intestine, liver and kidneys) [1–3]. P-gp is encoded by MDR1 in humans and by *mdr1a* and *mdr1b* in rodents. Based on the *mdr1a/b*^(-/-) knockout mouse model, the function of P-gp at the BBB and BPB appears to be more important in drug disposition than in other organs [4,5]. The brain distribution of verapamil, a P-gp substrate, in *mdr1a/b*^(-/-) mice is approximately 10-fold greater than that observed in the wild-type mice [4–6]. Likewise, the fetal distribution of the anti-HIV protease inhibitor, saquinavir, in the *mdr1a/b*^(-/-) fetuses is 20-fold greater than that in the wild-type fetuses [7]. Based on these data, it is widely presumed that P-gp activity at the human BBB and the BPB is important in restricting the distribution of drugs into the brain and fetus respectively. However, it is not ethically possible to measure the distribution of drugs into human brain or fetal tissues *in vivo*. Unlike the *mdr1a/b*^(-/-) model, determining P-gp activity at the BBB or BPB in larger animals is difficult due to the cost of obtaining brain and fetal tissue samples at multiple time points. Thus, a highly sensitive imaging method is required that can non-invasively measure the brain (human) and fetal (large animals) tissue concentrations of drugs that are P-gp substrates.

An elegant and non-invasive method to examine P-glycoprotein (P-gp) activity at the BBB, BPB and other organs is positron emission tomography (PET) using [¹¹C]-verapamil as the P-gp substrate [8–11]. Although verapamil is an excellent P-gp substrate (well-established, approved for human IV administration and relatively safe), it suffers from the disadvantage of undergoing extensive hepatic metabolism by cytochrome P450 (CYP) enzymes (Fig. 1) [9, 12–14].

Multiple investigators have used high performance liquid chromatography (HPLC) as a means of separating [¹¹C]-verapamil from its radiolabeled metabolites [15–17] with run times longer than 15 minutes. However, since [¹¹C] activity half-life is short (~20 min.), it is difficult to conduct rigorous HPLC quantification of the content of [¹¹C]-verapamil and its various metabolites in numerous blood or plasma samples obtained during a PET imaging study. Therefore, to rapidly quantify the content of [¹¹C]-verapamil and its metabolites circulating in the plasma, in both humans and non-human primates (*M. nemestrina*), we developed and validated a rapid solid-phase extraction method to separate [¹¹C]-verapamil from its major circulating metabolites.

2. Materials and Methods

2.1. Chemicals

SPE C₈ cartridges (1cc, 100mg) were purchased from Varian Inc, Lake Forest, CA. Verapamil was obtained from Sigma Aldrich (St Louis, MO). D-617, D-717, D-702 and D-703 were generously supplied by Knoll AG (Ludwigshafen, Germany) and Prof. W. L. Nelson (Medicinal Chemistry, University of Washington, Seattle). All other reagents were of the highest grade available from commercial sources.

2.2. PET imaging studies in healthy human volunteers and pregnant *M. nemestrina*

The healthy human volunteer study has been previously described in detail by Sasongko et al [11]. Briefly, after obtaining approval from local regulatory committees, [¹¹C]-verapamil (approx. 7.4 MBq/kg) was infused intravenously in a total volume of 5 to 10 mL over 1 min to six male and six female volunteers prior to and after at least one hour of infusion of the P-gp inhibitor, cyclosporine A (2.5 mg/kg/hr). All human subjects signed informed consent prior to participation in the study. Before the onset of the infusions and after each [¹¹C]-verapamil administration, multiple blood samples were obtained and the plasma was isolated. Plasma samples from 0, 1, 5, 10, 15, 20 and 45 min post injection were assayed by SPE and the 5 and

20 min samples were also assayed by HPLC. The protocol for the pregnant *M. nemestrina* subjects (n=6; gestational age 55–159 days), approved by the local animal care committee, was identical to that described above for humans except that 13.7 to 68.9 MBq/kg of [^{11}C]-verapamil was administered. Then, plasma samples were obtained at 1, 4, 14, 20, and 40 min, processed by SPE, and those obtained at 4 and 20 min samples were also assayed by HPLC.

2.3. Sample preparation and solid phase extraction for validation

Validation of the extraction process was conducted as if radiolabeled samples were acquired in real time from a PET study. Prior to extraction, each SPE cartridge was conditioned with 1 mL of 0.1 M hydrochloric acid (HCl):acetonitrile (10:90) v/v; 5 mM ammonium acetate [pH 4.2]/acetonitrile (70:30) v/v followed by 1 mL of PBS. Blank human plasma (980 μL) was spiked with 20 μL of a mixture of unlabeled verapamil, D-617, D-717, D-702 and D-703 (5 μg each) to make up a total volume of 1 mL. An aliquot (900 μL) of the spiked plasma was loaded onto SPE cartridge. Vacuum was applied and maintained at about 6 mm Hg to elute the plasma into a culture tube containing 1 mL acetonitrile, fraction 1 [F1]. Sequential elution was conducted with the following solvents: 1 mL PBS, fraction 2 [F2]; 3 mL of 5 mM ammonium acetate [pH 4.2]:acetonitrile (70:30) v/v, fraction 3 [F3]; 1 mL of 5 mM ammonium acetate [pH 4.2]:acetonitrile (70:30) v/v, fraction 4 [F4]; 1 mL of 0.1 M HCl:acetonitrile (10:90) v/v, fraction 5 [F5] (Figure II). The extraction process was completed in less than 5 minutes per sample and multiple samples could be extracted at the same time.

2.4. Sample preparation and extraction for PET study samples

Samples for the PET study were processed as described above (Section 2.3) except that the PET subject plasma sample (980 μL) was spiked with 20 μL of a mixture of unlabeled verapamil and D-617 (5 μg of each). After SPE elution, each fraction was counted on a gamma counter (Cobra Counter; Packard Corporation, Meriden, Conn). The background was subtracted from the sample counts and the resulting values decay corrected.

2.5. HPLC validation of the SPE procedure

To validate the specificity of the above SPE method, HPLC analysis of selected PET study samples (5 and 20 min for humans and 4 and 20 min for *M. nemestrina*) was conducted and compared with the results obtained with the SPE method. The subject's plasma (500 μL) was spiked with non-labeled analytes (5 μg of verapamil and D-617), deproteinized with equal amount of acetonitrile (500 μL), and centrifuged (45 g) for 3 minutes at room temperature. 100 μL of the supernatant were counted and 500 μL were directly injected onto the HPLC and eluted using the conditions described below (Section 2.6). Fractions of the eluent were collected every 20 seconds for 25 minutes and counted for [^{11}C] activity. Retention times of the unlabeled analytes were used to identify the collected fractions. The total counts associated with each analyte were expressed as percent of those injected onto the HPLC. These values were then compared with those obtained using the SPE method.

2.6. Recovery

Samples corresponding to the above SPE extracted samples were injected onto the HPLC-UV (Section 2.7), analyzed, compared with the SPE extracted samples to determine recovery of the analytes in the SPE fractions. This was performed in triplicate on multiple days. By spiking actual PET plasma samples with non-radiolabeled analytes prior to SPE, recovery of the radiolabeled compounds could be determined at a later date based on the assumption that the radiolabeled compounds extract with similar efficiency as the unlabeled compound. Recovery of the unlabeled compound could then be used to correct and calculate [^{11}C] activity from each fraction as verapamil [F5] or D-617 [F3 & F4], and the remainder was labeled as unknown "polar" metabolite(s) [F1 & F2].

2.7. High Performance Liquid Chromatography

Separation and detection of D-617, D-717, D-702/703 (baseline separation of D-702 and D-703 was not achieved), and verapamil was achieved on a Waters Alliance 2695 HPLC using a Zorbax SB-C₁₈ (250 × 4.6mm, 5μm; Agilent Technologies, Palo Alto, CA) column coupled with a Waters 2996 photodiode array detector (PDA) set at wavelength of 229 nm. Compounds were eluted at a flow rate of 1 mL/min using a gradient of 30 mM ammonium acetate (pH 5.0) and acetonitrile (mobile phase A and B, respectively). The gradient was adjusted linearly as follows: 85% A: 15% B held for 3 minutes, adjusted to 50% A: 50% B over the next 6 minutes, further decreased to 10% A: 90% B over 4 minutes, held for 1 minute, then rapidly returned to 85% A: 15% B over the next 0.5 minute. 5 minutes was allowed for re-equilibration prior to next injection.

3. Results

3.1. High Performance liquid chromatography

An example of a typical HPLC-UV chromatogram generated using the conditions described above is shown in Figure III. Retention times were 7.9, 8.6, 9.8/9.9, and 10.5 min for D-717, D-617, D-703/702, and verapamil, respectively, for a total run time of 20 min (Figure III). The total run time could be shortened by increasing the gradient and dwell time. However, an initial increase in the gradient tended to decrease baseline separation between D-617 and D-717. We were able to achieve baseline separation for all analytes except for D-702 and D-703. Based on expected circulating metabolites (D-617 and polar metabolites) reported in the literature and our preliminary analysis of PET human and *M. nemestrina* plasma samples, our HPLC samples were spiked with only verapamil and D-617 to determine their recovery from SPE extraction in actual PET study samples (see Discussion).

3.2. Solid phase extraction (SPE) and recovery

The recovery from spiked human plasma samples was validated at least in triplicate for inter-day variability. Greater than 95% of verapamil and D-617 were recovered from the acetonitrile-HCl [F5] and ammonium acetate fractions [F3], respectively (Table I). A minor cross contaminant D-617 (2.6%) was observed in the acetonitrile-HCl fraction [F5]. Other metabolites that are known to carry the [¹¹C] radiolabel were also tested for their percent recovery. D-717, a minor O-demethylated product of D-617 was also extracted from the ammonium acetate fraction [F3] with more than 85% recovery. In addition, O-demethylation of [¹¹C] verapamil leads to D-702 and D-703 formation and these two metabolites were eluted in the acetonitrile-HCl fraction [F5] with recovery of 83.4% and 92.8%, respectively (Table I). The secondary wash step of ammonium acetate [F4] was used to minimize sample contamination from D-617 into the verapamil fraction [F5]. Less than 2% of D-617 was observed in this wash step and was included in the recovery, by combining fractions F3 and F4. Verapamil, D-617, D-717, D-702, or D-703 were not detected in the PBS fraction [F2] or the initial plasma loading fraction [F1]. Recovery from PET plasma samples demonstrated >90% recovery for verapamil and D-617 for both human and *M. nemestrina* samples (Table II).

3.3. HPLC validation of the SPE results

Direct HPLC analysis of plasma samples obtained from human or *M. nemestrina* dosed with [¹¹C]-verapamil, showed a lack of presence of [¹¹C]-D-702, [¹¹C]-D-703 and [¹¹C]-D-717. In addition, there was no significant difference (paired t-test, p<0.05) in analyte quantification of [¹¹C]-verapamil and [¹¹C]-D-617 between the SPE and HPLC analysis of the same plasma sample (Figure IV A & B and Table III).

4. Discussion

Verapamil is metabolized to numerous metabolites in the body (Figure I). Of these, only those that retain the N-methyl group will be radioactive. For example, norverapamil, a major metabolite, formed by N-demethylation, will not be radioactive and will not confound interpretation of the radioactivity present in the tissues or plasma. Therefore, we focused our SPE method development on rapidly separating the radioactive metabolites that are present in both human and macaque plasma. To ascertain the latter, we determined by HPLC the radioactive contents of the plasma samples at 20 min after [^{11}C]-verapamil administration to humans and macaques. The 20 min time point was chosen because the concentrations of the metabolites in plasma at this time were likely to be significant. The HPLC method was capable of separating verapamil from all of its N- and O-demethylated metabolites. Using HPLC, we found that the circulating metabolites present in both human and macaque plasma were D-617 and those that eluted in the solvent front. We did not observe the presence of other N- and O-demethylated metabolites ([^{11}C]-D-702, [^{11}C]-D-703 or [^{11}C]-D-717) in these plasma samples, nor did any of these metabolite(s) elute in the solvent front. For this reason, our SPE method did not need to separate D-717 from D-617 or D-702/3 from verapamil. However, it did separate verapamil from the relevant metabolites, namely the polar metabolite(s), and D-617. There was very little cross-contamination of these relevant metabolites or verapamil in the fractions corresponding to each of these entities. Moreover, the recovery of D-617 and verapamil was >90% for both analytes from both study groups (humans and *M. nemestrina*, Table II). Recovery of the polar metabolite(s) could not be computed as its identity is unknown and a pure standard for this metabolite(s) is not available.

When the SPE method was applied to plasma samples obtained from PET studies conducted in humans and macaques, we found an identical metabolic profile of verapamil in both species. That is, only D-617 and the polar metabolite(s) were found circulating in the plasma. Others [15,18] have also confirmed the lack of the O-demethylated metabolites in human plasma. In contrast, we have found that the rat does not have significant quantity of D-617 or any other O- or N-demethylated metabolites circulating in the plasma after administration of radiolabeled verapamil [8]. However, we did find the polar metabolite(s) circulating in the rat plasma. This metabolite(s) has also been reported by others in humans and rats [8,10,11]. The identity of this metabolite(s) is not known. Luurtsema et al. [10] have speculated that these metabolites are [^{11}C]-formaldehyde and its subsequent metabolic products. In addition, some of the metabolites may be polar conjugates of the O-demethylated products of verapamil. We have confirmed that non-radiolabeled formaldehyde is eluted in the PBS fraction in our SPE system and elutes with the polar metabolite(s) in our HPLC system.

There was significant concordance between the SPE and HPLC method in the quantification of verapamil, D-617 and the polar metabolite(s). This concordance indicates that the SPE method can be used to quantify the circulating metabolites in plasma. The decline of verapamil plasma concentration in the macaque is faster than that in humans indicating more rapid distribution and/or metabolism of verapamil in the former (See Figure IV A&B). In order to reduce the complication of metabolites confounding the interpretation of imaging data, it is important to keep the metabolism of [^{11}C]-verapamil to a minimum. This allows PET images of tissues to be interpreted as only [^{11}C]-verapamil rather than [^{11}C]-verapamil plus metabolites. Interestingly, the major circulating metabolite of verapamil, D-617, is also a P-gp substrate [19]. Thus, its behavior will likely mimic that of verapamil. That is, D-617 will also be excluded from tissues expressing high activity of P-gp. Hence, its presence in the plasma and therefore the tissue can be viewed as being equivalent to [^{11}C]-verapamil and should not confound the interpretation of tissue PET images. When both the human and macaque verapamil metabolic profiles in plasma are examined up to 20 and 10 min after [^{11}C]-verapamil administration respectively, the majority of the radioactivity in the plasma (>80% for both) is

associated with verapamil and D-617. Thus, PET images of tissues up to these time points can be interpreted as constituting mostly [^{11}C]-verapamil. Implicit in this assumption is the notion that verapamil is not extensively metabolized in extrahepatic tissue, such as the brain [20, 21].

In conclusion, we have developed a rapid SPE method to separate [^{11}C]-verapamil from its biologically relevant radioactive metabolites found in human and macaque plasma. This method allows real-time and rapid analysis of plasma samples obtained during a [^{11}C]-verapamil imaging study not possible with conventional HPLC separation. Such a SPE method is necessary for [^{11}C]-verapamil PET imaging studies to ascertain the extent of metabolism of [^{11}C]-verapamil, given the short half life of [^{11}C]. This information is important for correctly interpreting tissue P-gp activity using PET.

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Figure I. Metabolic pathway of verapamil. In humans and *M. nemestrina*, only verapamil, D-617, and the polar metabolites (identity unknown) were detectable.

Figure II.
Elution sequence of verapamil and its major metabolites using Stable Bond C₈ Solid Phase Extraction (SPE) cartridges.

Figure III.
HPLC chromatogram of D-717, D-617, D-703, D-702, and verapamil

Figure IV.

The change with time in percent content of [^{11}C]-verapamil, [^{11}C]-D-617/717 and [^{11}C]-polar metabolites in plasma as determined by SPE in (A) healthy human subjects ($n = 11$) or (B) pregnant *M. nemestrina* ($n=6$) administered [^{11}C]-verapamil. Results are mean $\pm$ S.D.

Table I
Recovery of verapamil and metabolites (5 µg) individually spiked in blank human plasma and extracted using C₈ SPE cartridges.

Fraction	Verapamil*	Inter-day Mean Percent Recoveries ± S.D.		
		D-617*	D-717*	D-702* D-703*
PBS [F2]	ND	ND	ND	ND ND
Ammonium acetate [F3+F4]	ND	98.5 ± 7.7	85.4 ± 4.6	ND ND
HCl-Ammonium acetate [F5]	96.6 ± 4.5	2.6 ± 0.8	ND	83.4 ± 5.3 92.8 ± 6.5

ND-Not detected, PBS- phosphate-buffered saline

* Mean ± S.D. of 3 or 4 independent experiments performed in either triplicates or quintuplets in 4 independent experiments with individual analytes.

Table II

Percent recovery of spiked verapamil and D-617 from human (n=11) or *M. nemestrina* (n=6) plasma obtained at multiple time points during verapamil PET imaging study.

	Inter-day Mean Percent Recoveries $\pm$ SD (range)	
	Verapamil	D-617
Human	91 $\pm$ 9.4 (79–99)	90 $\pm$ 13 (71–100)
<i>M. nemestrina</i>	95 $\pm$ 11 (73–113)	94 $\pm$ 8.4 (74–111)

The percent content of [¹¹C]-analyte in human (n=11) and *M. nemestrina* (n=6) plasma samples obtained during PET imaging and assayed simultaneously by SPE and HPLC

Table III

	Time (min)	Percent [¹¹ C]-Analyte (mean ± SD)					
		Verapamil		D-617717		Polar Metabolite (s)	
		SPE [*]	HPLC [*]	SPE [*]	HPLC [*]	SPE [*]	HPLC [*]
Human	5	94.9±6.8	94.0±7.6	3.5±8.7	1.0±1.5	2.1±0.9	1.2±1.0
	20	66.9±8.9	61.2±9.9	14.7±6.1	12.4±8.3	17.4±9.3	13.8±6.8
<i>M. nemestrina</i>	4	71.8±18.4	81±7.2	12.9±5.2	10±7.4	11.6±6.7	9.67±4.8
	20	24.2±6.7	29±5.3	40±7.7	40.5±18.8	47.5±7.1	42.2±12.8

* Quantification of verapamil and its metabolites by SPE and HPLC were not significantly different (p > 0.05) as determined by a paired t-test.