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Non-invasive *in vivo* imaging of acute thrombosis: development of a novel factor XIIIa radiotracer

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Aims

Cardiovascular thrombosis is responsible a quarter of deaths annually worldwide. Current imaging methods for cardiovascular thrombosis focus on anatomical identification of thrombus but cannot determine thrombus age or activity. Molecular imaging techniques hold promise for identification and quantification of thrombosis *in vivo*. Our objective was to assess a novel optical and positron-emitting probe targeting Factor XIIIa (ENC2015) as biomarker of active thrombus formation.

Methods and results

Optical and positron-emitting ENC2015 probes were assessed *ex vivo* using blood drawn from human volunteers and passed through perfusion chambers containing denuded porcine aorta as a model of arterial injury. Specificity of ENC2015 was established with co-infusion of a factor XIIIa inhibitor. *In vivo* ¹⁸F-ENC2015 biodistribution, kinetics, radiometabolism, and thrombus binding were characterized in rats. Both Cy5 and fluorine-18 labelled ENC2015 rapidly and specifically bound to thrombi. Thrombus uptake was inhibited by a factor XIIIa inhibitor. ¹⁸F-ENC2015 remained unmetabolized over 8 h when incubated in *ex vivo* human blood. *In vivo*, 42% of parent radiotracer remained in blood 60 min post-administration. Biodistribution studies demonstrated rapid clearance from tissues with elimination via the urinary system. *In vivo*, ¹⁸F-ENC2015 uptake was markedly increased in the thrombosed carotid artery compared to the contralateral patent artery (mean standard uptake value ratio of 2.40 vs. 0.74, $P < 0.0001$).

Conclusion

ENC2015 rapidly and selectively binds to acute thrombus in both an *ex vivo* human translational model and an *in vivo* rodent model of arterial thrombosis. This probe holds promise for the non-invasive identification of thrombus formation in cardiovascular disease.

Keywords

Positron emission tomography • Thrombus • Thrombosis • Atherothrombosis • Radiotracer • PET/CT

Introduction

Acute vascular thrombosis is common to the pathogenesis of myocardial infarction, stroke, and venous thromboembolism which collectively are responsible for a quarter of all deaths annually worldwide.¹ At present, imaging approaches to the detection of

acute thrombosis consist mainly of thrombus visualization with ultrasound, invasive catheterization, computerized tomography (CT), and magnetic resonance angiography. Common to all of these imaging modalities is a lack of sensitivity and specificity for acute thrombus detection.^{2–4} None can accurately ascertain whether a thrombus is physiologically active or dormant which can

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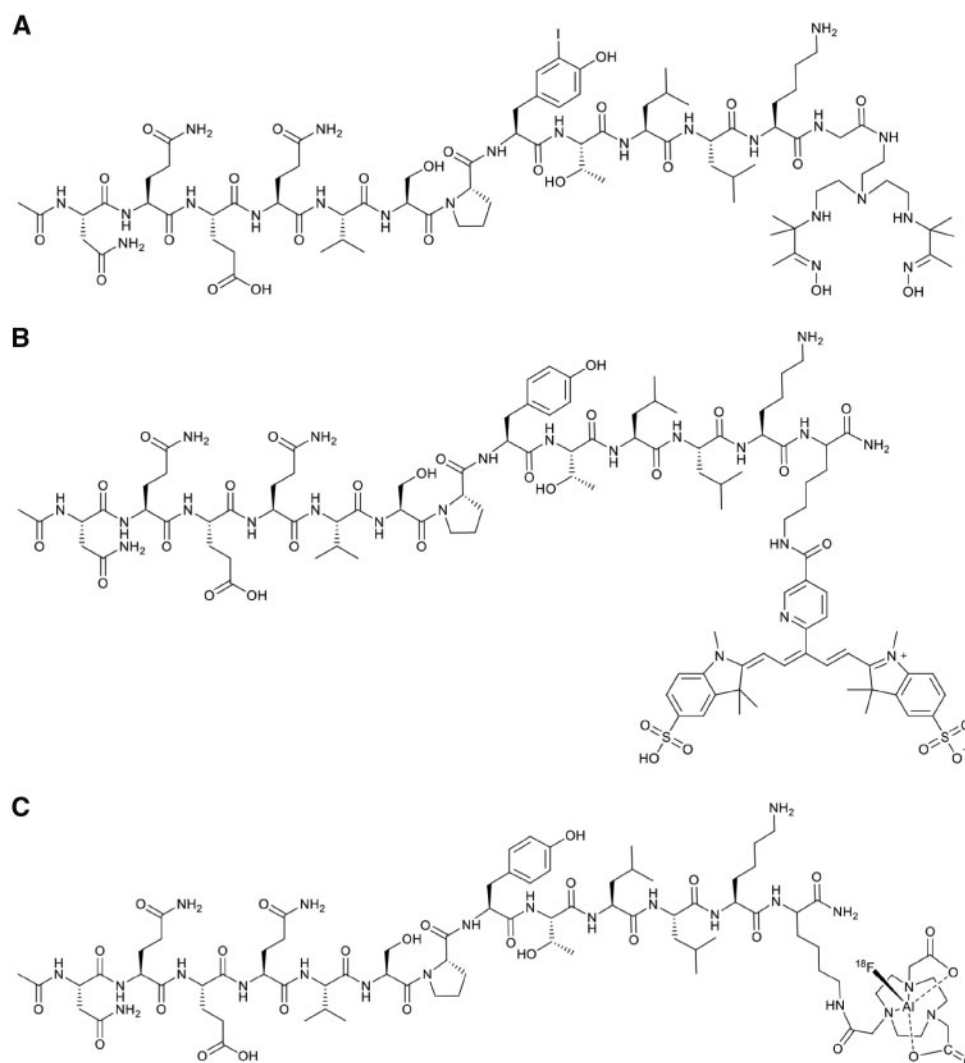


Figure 1 Cy5 and fluorine-18 labelled chemical structure of ENC2015 compound. (A) The chemical structure of NC100668 upon which ENC2015 is based.^{8,9} (B) The ENC2015 peptide with the optical Cy5 label. (C) The ENC2015 peptide after chelation with Al^{18}F .

make diagnostic and management decisions regarding anti-thrombotic therapy difficult. These limitations support the development of novel imaging techniques to detect acute vascular thrombosis with greater precision.⁵

Molecular positron emission tomography (PET) imaging offers the potential to address a number of these issues through visualization and quantification of physiological thrombus activity.⁵ Thrombus tracers have so far shown promise in monitoring thrombolysis efficacy in rats⁶ and the detection of acute deep vein thrombosis and pulmonary embolism in humans.⁷ However, none of these PET radiotracers targets the coagulation factor XIIIa (FXIIIa). In the present study, we report on the development of a novel factor XIIIa-directed optical and PET radiotracer, ENC2015 (Figure 1),^{8,9} for the non-invasive imaging of thrombosis *in vivo*. ENC2015 is based on $^{99\text{m}}\text{Tc}$ -NC100668, a small single-photon emission computerized tomography (SPECT) FXIIIa-targeted radiotracer. The coagulation factor FXIIIa acts to strengthen fibrin crosslinking in newly formed

thrombus, thus serving as a valuable target to assess thrombus activity and age. Previously, binding of NC100668 to thrombus has been demonstrated in *in vitro* plasma clot assays, a rat model of inferior vena cava thrombosis, and human autopsy specimens of pulmonary thromboemboli.^{10,11} In contrast to NC100668, ENC2015 is labelled with the PET radioisotope fluorine-18 thus allowing for clinical imaging datasets with higher resolution and improved quantification compared with SPECT. Furthermore, ENC2015 was developed as both an optical near-infrared fluorescent probe and an ^{18}F positron-emitting radiotracer to image thrombosis *in vivo*. A dual optical-PET labelled probe would hold great promise for *in vivo* diagnosis and histological tissue validation with a single chemical moiety. Moreover, ENC2015 offers the potential to allow identification of disease before clinical manifestation, track thrombus activity over time, and monitor therapeutic efficacy. Here, we report our pre-clinical assessment of ENC2015 in an *ex vivo* model of human thrombosis and an *in vivo* rodent carotid thrombosis model.

Methods

Synthesis of ENC2015

The peptide scaffold of ENC2015 was prepared by Fmoc solid-phase peptide synthesis using Rink amide Chemmatrix resin and HBTU/DIPEA assisted couplings, monitored by Kaiser ninhydrin test and regular test cleavages followed by HPLC and MALDI-TOF MS analyses. After the assembly of the peptide and capping with Ac_2O , the ϵ -amine Mmt protecting group on the Lysine residue at the C-terminus was deprotected on resin and derivatised with sulfo Cy5 using PyBop as a coupling reagent. Cy5-ENC2015 was obtained after cleaving off the peptide from the solid support and semi-preparative HPLC purification in low yield (4% overall yield) but excellent purity (>98% at 210 nm). The peptide precursor of Al^{18}F -ENC2015 [peptide with *p*-SCN-Bn-1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) chelator] was prepared by Fmoc solid-phase peptide synthesis using Rink amide Chemmatrix resin and HBTU/DIPEA assisted couplings, monitored by Kaiser ninhydrin test and regular test cleavages followed by HPLC and MALDI-TOF MS analyses. After the assembly of the peptide and capping with Ac_2O , the ϵ -amine Dde protecting group on the Lysine residue at the C terminus was de-protected on resin and derivatised with NOTA-NHS. The precursor was obtained after cleavage from the solid support and purifying it by semi-preparative HPLC purification in low yield (5% overall yield) but excellent purity (>97% at 210 nm).

Radiolabelling of ENC2015

The radiosynthesis of ^{18}F -ENC2015 was performed at Edinburgh Imaging Facility on a commercial synthesizer, GE TRACERlab[®] MX. A disposable kit was assembled to allow the peptide conjugate to chelate with aluminium fluoride and undergo purification using an automated method. Purified Fluoride-18 (using SepPak QMA Light cartridge eluted with 300 μL saline, ca. 10 GBq) was added to a mixture of NOTA-peptide (12 μL , 2 mM solution in NaOAc buffer pH 4) in the presence of AlCl_3 (6 μL , 2 mM in NaOAc buffer pH 4). Acetonitrile was used as an organic co-solvent (aqueous solution to organic solvent ratio of 1:1.2 v/v) and the mixture was heated to 100°C for 10 min. The reaction mixture was diluted with water (20 mL) and passed through a SepPak Light C18 cartridge (pre-conditioned with ethanol and water). The C18 cartridge was washed with water (2 \times 20 mL) and product was eluted with ethanol (1.5 mL). The final product was formulated in a physiological solution containing 10% ethanol in normal saline. ^{18}F -ENC2015 was obtained with an average non-decay corrected yield of $27 \pm 11\%$ (starting from 10 ± 6 GBq of fluoride-18, $n = 15$) after a total synthesis time of 28 min. Identity of ^{18}F -ENC2015 and its radiochemical purity (>99%) were determined by analytical HPLC (C18, 250 \times 4.6 mm, 1 mL/min, UV absorbance at $\lambda = 267$ nm) at the end of the synthesis. ^{18}F -ENC2015 specific activity was >45 GBq/ μmol .

Ex vivo translational model of human thrombus formation

To confirm target engagement, we examined binding of ENC2015 to newly formed thrombus using an *ex vivo* translational model of acute thrombus formation (Supplementary data online, Figure S1) as described previously.^{12–15} In brief, a pump (Masterflex model 7013, Cole-Palmer Instruments, USA) was used to draw native (un-anticoagulated) blood from the antecubital vein of healthy volunteers through a series of three cylindrical perfusion chambers maintained at 37°C in a water bath. Each chamber contained a carefully prepared strip of porcine aorta (Pel-Freez Biologicals, CA, USA) from which the intima and a thin layer of media had

been removed. Rheological conditions in the first chamber simulated those of patent medium-sized coronary arteries (low-shear rate, 212 s^{-1}), whereas those in the second and third chambers simulated those of mild to moderately stenosed coronary arteries (high shear rate, 1690 s^{-1}). Each study lasted for exactly 5 min during which flow was maintained at a constant rate of 10 mL/min. Following perfusion of blood, the chambers were perfused with 0.9% saline for one minute under the same rheological conditions. Thereafter, the porcine strips with thrombus attached were removed and fixed in 4% paraformaldehyde for 24 h at 4°C prior to being prepared for histological analysis.

Sixteen healthy male volunteers (31 ± 1.26 years) were enrolled into the study which was performed with the approval of the local research ethics committee (AMREC; 17-HV-033), in accordance with the Declaration of Helsinki, and the written informed consent of all the volunteers. Exclusion criteria were history of bleeding diathesis, use of regular medication or any clinically significant illness. We conducted the study in a series of sequences: (i) infusion of Cy5-ENC2015 (250 nM, $n = 6$) or ^{18}F -ENC2015 (4.6 ± 0.5 MBq/mL, $n = 10$); (ii) infusion of Cy5-ENC2015 (250 nM, $n = 6$) or ^{18}F -ENC2015 (5.5 ± 0.9 MBq/mL, $n = 10$) plus factor XIIIa inhibitor (Iodoacetamide, Sigma-Aldrich, UK) at a concentration 10 mg/mL; and (iii) blood control without infusion of optical or radiolabelled tracer ($n = 6$). For the optical probe, further control experimental groups included: (iv) the administration of ENC2015 in the absence of blood, (v) the infusion of free Cy5 fluorophore, and (vi) the infusion of a scrambled peptide sequence (Supplementary data online, Figure S2B) in the presence of thrombus. In one volunteer, we collected blood through the chamber for HPLC radiometabolite analysis to assess tracer stability in *ex vivo* human blood at 0 and 8 h after radiotracer incubation. Furthermore, paraffin-embedded cross-sections of *ex vivo* human thrombi ($n = 3$) were prepared for autoradiographic experiments performed in quadruplicate; (i) ^{18}F -ENC2015; (ii) ^{18}F -ENC2015 co-incubated with homologous unlabelled 'cold' ENC2015 (5 μM); (iii) ^{18}F -ENC2015 co-incubated with heterologous scrambled tracer (5 μM); and (iv) ^{18}F -ENC2015 co-incubated with iodoacetamide (5 μM), a non-selective irreversible inhibitor of all cysteine peptidases, which in this experimental design works as negative control. All tissue sections were de-paraffinized, rehydrated and incubated for 2 h at 37°C, and then slides were washed twice with phosphate buffer solution before being air-dried and exposed to a Fujifilm BAS-IP MS 2040 phosphor screen (Fujifilm, Japan).

Porcine aortic strips from the Badimon chamber experiments were imaged for macroscopic (Biospace Lab PhotonIMAGER Optima system, France) and microscopic thrombus fluorescence (Zeiss Axio Scan.Z1, Germany) on the Cy5 channel (645/670 nm wavelength excitation and emission). Strips exposed to ^{18}F -ENC2015 were imaged with the nanopositron emission tomography-computed tomography (NanoPET/CT, Mediso, Hungary) enabling quantification of tracer uptake. Phosphor screens were imaged on a Fujifilm FLA5100 imaging plate reader (Fujifilm, Japan).

Animal studies

All experiments were conducted in accordance with institutional animal care protocols, authorized by the Home Office under the Animals (Scientific Procedures) Act 1986 and conformed to EU guidelines (Directive 2010/63/EU) of the European Parliament on the protection of animals used for scientific purposes. Adult male rats (16.7 ± 3.8 weeks, 392.8 ± 74.1 g, $n = 8$) were anaesthetized with 2–2.5% isoflurane (50/50 oxygen/nitrous oxide, 1 L/min). Body temperature was maintained by heated scanner bed or heated mat and monitored by a rectal thermometer. Vital signs including heart rate and respiration rate were monitored continuously during the experiments. Biodistribution was assessed in

Table 1 Optical tracer uptake in *ex vivo* translational model of human thrombus formation

Sequence	ENC2015 + blood (Sequence 1)	ENC2015 + blood + inhibitor (Sequence 2)	Blood only (Sequence 3)	P value (1 vs. 2)	P value (1 vs. 3)
fluorescence					
Low shear macroscopic (ph/s/cm ² /sr)	$4.4 \times 10^7 \pm 0.89 \times 10^7$	$0.33 \times 10^7 \pm 0.13 \times 10^7$	$1.16 \times 10^7 \pm 0.67 \times 10^7$	0.01	0.01
Low shear microscopic (AU)	7307 ± 403	3297 ± 30	3103 ± 48	<0.0001	<0.0001
High shear macroscopic (ph/s/cm ² /sr)	$6.38 \times 10^6 \pm 2.3 \times 10^6$	$1.22 \times 10^6 \pm 0.45 \times 10^6$	$1.24 \times 10^6 \pm 0.67 \times 10^6$	0.09	0.05
High shear microscopic (AU)	4966 ± 118	3356 ± 53	3078 ± 29	<0.0001	<0.0001

Each row represents thrombus type and quantification method used to analyse tracer uptake with each column representing the constituents of each experimental run. Tracer uptake in Sequence 1 (blood + tracer) was significantly greater than 2 (blood + tracer + inhibitor) and 3 (blood only) in all thrombi bar the macroscopically analysed high shear thrombi. Data are shown as mean ± SEM ($n = 6$; paired two-way ANOVA).

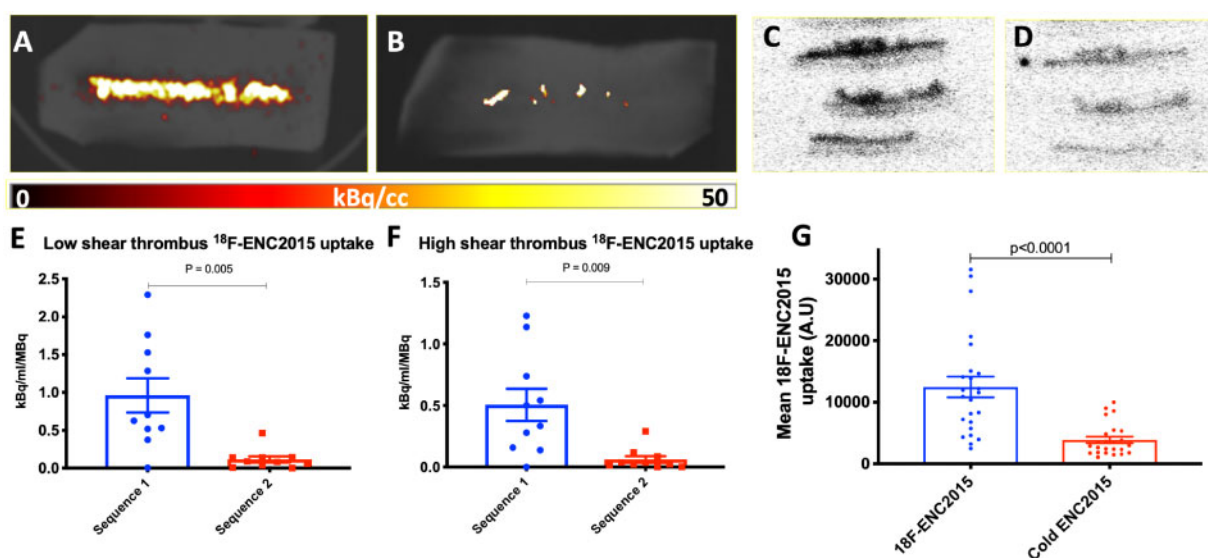


Figure 4 Binding of ¹⁸F-ENC2015 to acutely formed human thrombus. (A) Avid thrombus binding of ¹⁸F-ENC2015 on Micro-PET CT similar to optical version of ENC2015. (B) Diminished thrombus uptake of the radiotracer in the presence of factor XIIIa inhibitor. (C) An autoradiographic plate showing uptake of ¹⁸F-ENC2015 on sections (5 μm thickness) of freshly formed human thrombus. (D) Corresponding serial sections pre-treated with cold tracer demonstrating specific inhibition of binding sites. Bar charts (E + F) demonstrate ¹⁸F-ENC2015 tracer uptake in both low and high shear thrombi (Sequence 1) and how this is specifically blocked by a factor XIII inhibitor (Sequence 2) ($n = 10$). (G) Bar chart shows a marked and significant reduction in ¹⁸F-ENC2015 uptake in human thrombus co-incubated to unlabelled tracer ($n = 3$). Values expressed as mean ± SEM, (paired t-test).

radiometabolite (Figure 5A–D). In all animals, ¹⁸F-ENC2015 was rapidly cleared from the vascular bed, heart, and lungs and was predominantly excreted via the kidneys (Supplementary data online, Figure S5). The mean percentage of total injected ¹⁸F-ENC2015 in each major organ over time can be seen in Table 2. Radiometabolite analysis of *ex vivo* human blood using HPLC found the parent ¹⁸F-ENC2015 compound to remain unmetabolized after 8 h post-incubation (Supplementary data online, Figure S3).

¹⁸F-ENC2015 binds to thrombus *in vivo*

Rapid and high uptake of ¹⁸F-ENC2015 was observed in the carotid thrombus and skin incision site in all animals (Figure 6B and C). Mean thrombus SUVr began to surpass contralateral vessel SUVr 10 min

after injection. Thrombus SUVr peaked at 130 min with separation of the activity curves maintained after 180 min post-radiotracer administration (Figure 6C). Mean activity in the thrombosed vessel was higher than in contralateral vessel from 35 to 180 min ($P < 0.0001$, paired t-test).

Discussion

Here, we presented the synthesis and pre-clinical evaluation of ENC2015, a dual-purpose optical and PET Factor XIIIa targeted probe for detecting thrombi. In a translational model of deep vascular injury, we successfully demonstrated rapid and SB of ENC2015 to

human thrombus. Fluorescence and ^{18}F -ENC2015 uptake in low shear thrombi were markedly greater than in high shear thrombi which reflect greater fibrin deposition and factor XIIIa activity in acutely formed thrombi formed in a slow flowing venous system.^{18,19}

Table 2 Organ biodistribution of ^{18}F -ENC2015 over time

% ID	1 h	4 h
Heart	0.071 ± 0.016%	0.009 ± 0.001%
Lungs	0.061 ± 0.006%	0.014 ± 0.003%
Kidneys	16.542 ± 1.113%	14.938 ± 0.645%
Bladder	33.498 ± 4.769%	17.14 ± 3.564%
Liver	0.107 ± 0.025%	0.031 ± 0.015%
Brain	0.011 ± 0.001%	0.001 ± 0.0002%

Note the rapid reduction in decay corrected percentage of injected radioactivity from 1 h to 4 h in all analysed organs. Tracer accumulation was greatest in the kidneys owing to a renal site of metabolism and excretion. Data are shown as mean ± SEM ($n = 3$).

However, uptake of both probes was easily appreciable in the high shear, classically platelet-rich 'white thrombus'²⁰ illustrating the versatility of ENC2015 to identify both venous and arterial thrombi. To further enhance understanding of the relationship between fibrin deposition and platelet binding in both high and low shear thrombi, a comparison of ENC2015 with a platelet binding probe such as that reported by Kim *et al.*⁷ would be insightful.

Previous work on NC100668 deduced the major site of metabolism to be the renal cortex with a small fraction metabolized by proteases in the blood.^{8,11,21} Given the chemical similarity between the two peptides, it is likely that ENC2015 would undergo similar metabolic pathways as NC100668. Our analysis found the production of one major *in vivo* metabolite (Metabolite A, Figure 5, Graph C + D). This compound was only identified from *in vivo* rat blood sampling and was not found after 8 h of ^{18}F -ENC2015 incubation in *ex vivo* human blood. This demonstrates that uptake within human thrombus formed in the Badimon chamber studies must therefore be due to the activity of the parent compound and not a human blood-formed metabolite. Furthermore, it highlights the stability of

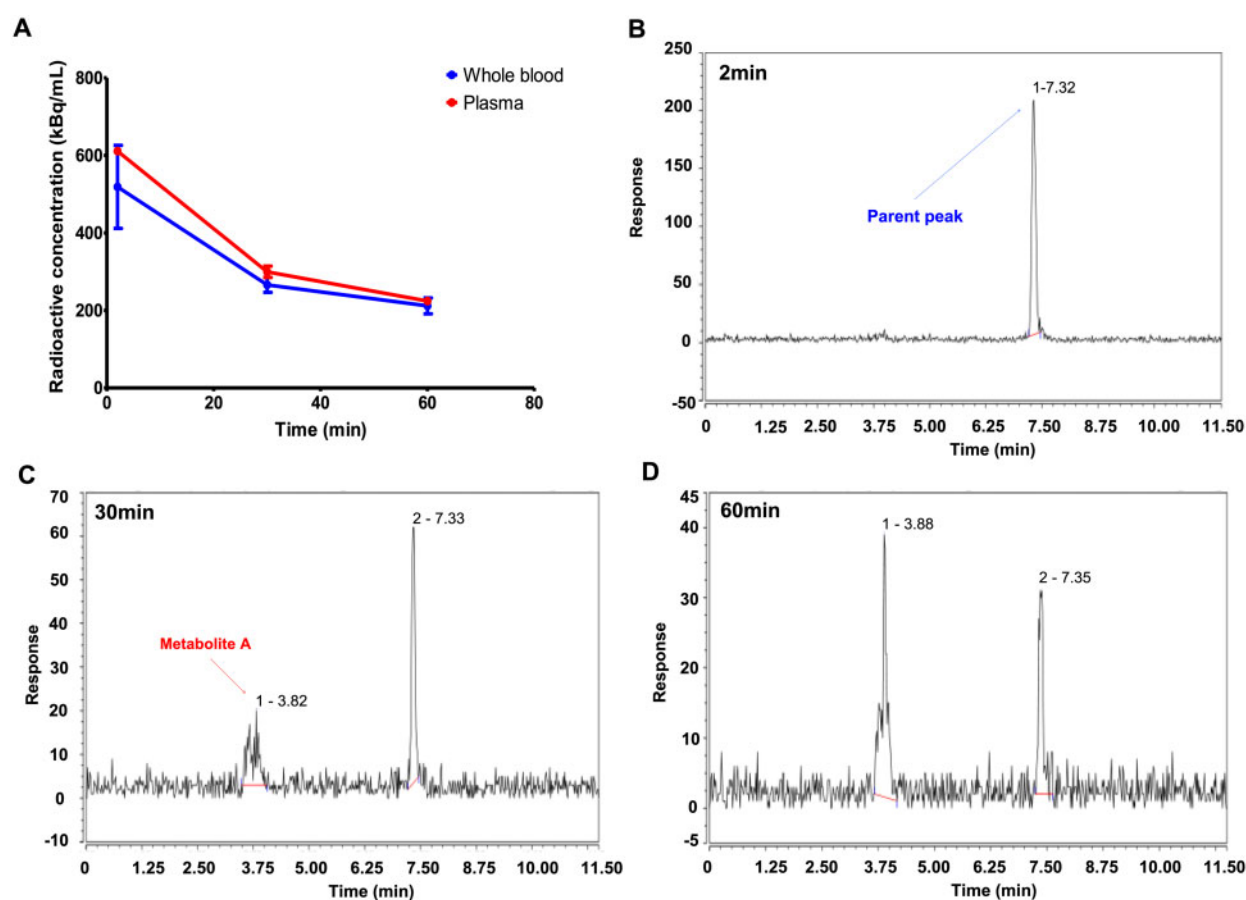


Figure 5 *In vivo* metabolism of ^{18}F -ENC2015 in rat blood. (A) Graph shows the radioactive concentration of ^{18}F -ENC2015 over time in rat whole blood and plasma ($n = 2$). (B) HPLC analysis in chromatogram shows a single peak of parent compound after 2 min of *in vivo* ^{18}F -ENC2015 circulation. (C) After 30 min, a second smaller peak 'metabolite A' can be appreciated on chromatogram. (D) At 60 min, chromatogram shows the radioactivity of metabolite A surpassing that of the parent compound.

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