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Diarmaid J. Murphy, Delphine Desjardins, Peter Boyd, Nathalie Dereuddre-Bosquet, Lev Stimmer, Anna Caldwell, Roger Le Grand, Charles Kelly, Jens van Roey, R. Karl Malcolm

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Diarmaid J. Murphy[a], Delphine Desjardins[b], Peter Boyd[a], Nathalie Dereuddre-Bosquet[b], Lev Stimmer[c], Anna Caldwell[d], Roger Le Grand[b], Charles Kelly[e], Jens van Roey[f] and R. Karl Malcolm[a]*

[a]*School of Pharmacy, Queen's University Belfast, Belfast BT9 7BL, UK* (d.j.murphy@qub.ac.uk, p.boyd@qub.ac.uk, k.malcolm@qub.ac.uk)

[b]*Université Paris Sud, INSERM, CEA, DRF-Immunology of Viral Infections and Autoimmune Diseases department (IMVA), U1184, IDMIT Infrastructure, Fontenay-aux-Roses, France* (delphine.desjardins@cea.fr, nathalie.bosquet@cea.fr, roger.le-grand@cea.fr)

[c]*Molecular Imaging Research Center, CEA-INSERM US27/U1169, 18 route du Panorama, 92265, Fontenay-aux-Roses, France* (lev.stimmer@cea.fr)

[d]*Mass Spectrometry Facility, King's College London, London SE1 9NH, UK* (anna.caldwell@kcl.ac.uk)

[e]*Dental Institute, Guy's Hospital, King's College London, London SE1 9RT, UK* (charles.kelly@kcl.ac.uk)

[f]*Janssen GPH, Turnhoutseweg 30, 2340 Beerse, Belgium* (JVROEY@its.jnj.com)

*Corresponding author. Tel: +44 (0)28 9097 2319; Fax: +44 (0)28 9024 7794;

E-mail: k.malcolm@qub.ac.uk

Running title: Impact of size and drug loadings on combination vaginal ring in cynomolgus macaques

Abstract

This work investigates the impact of vaginal ring size and drug loading on the *in vitro* release, safety, ease of fit, and pharmacokinetics in cynomolgus macaques of matrix-type silicone elastomer vaginal rings containing a combination of the non-nucleoside reverse transcriptase inhibitor dapivirine and the protease inhibitor darunavir. Drug-free and drug-loaded vaginal rings having three different geometries were manufactured by reaction injection molding. *In vitro* drug release was assessed using both a solvent/water mixture and a vaginal fluid simulant. Macaques fitted with drug-free vaginal rings for 28 days were assessed by colposcopy, cytological evaluation of cervico-vaginal lavage and histological evaluation of tissue after ring removal. The 20 × 4.5 mm combination ring, deemed most appropriate for vaginal fit and comfort in the macaques, was evaluted for pharmacokinetics over 28 days. Substantial differences were observed in the *in vitro* release profiles between the three ring sizes. However, these differences were not manifest *in vivo*, where measured drug concentrations after 20 × 4.5 mm ring use were not significantly different from those reported previously with a 25 × 6 mm ring. These results suggest that ring placement and fit is an important species-specific study parameter that should be optimised prior to pharmacokinetic testing.

Keywords: HIV microbicide; Silicone elastomer vaginal ring; Cynomolgus macaque

1. Introduction

Silicone elastomers have a long history of use in implantable drug delivery devices. During the early 1960s, reports first emerged that a wide range of substances – including anesthetic gases and steroid molecues – were able to permeate at near constant rates through the walls of silicone elastomer tubing (Dzuik and Cook, 1966; Folkman and Long, 1964; Long and Folkman, 1966),

and ultimately led to the development of the first contraceptive levonorgestrel-releasing subdermal contraceptive implant, Norplant® (Croxatto, 2002; Sivin et al., 1997).

The concept of a polymeric vaginal ring device for sustained or controlled drug administration to the human vagina for clinical benefit was first introduced in a series of patents and journal articles published in 1970 (Duncan, 1970; Mishell et al., 1970; Mishell and Lumkin, 1970). To date, six vaginal ring products – all offering release of one or more steroid molecules – have already reached market. Estring®, Progering®, Fertiring® and Femring® are manufactured from silicone elastomer materials for use in either contraception or hormone replacement therapy, while contraceptive ring products Nuvaring® and Ornibel® are manufactured from polyurethane and/or ethylene vinyl acetate thermoplastic polymers (Malcolm et al., 2016). Although individual ring products are offered in a single size to fit most women, the overall diameter (50–60 mm) and cross-sectional diameter (4–9 mm) varies between products and depends upon the type of polymer and the ring design.

A number of vaginal ring devices are being developed for sustained release of antiretrovirals for prevention of sexual transmission of human immunodeficiency virus type 1 (HIV-1) (Malcolm et al., 2012a, 2016; Thurman et al., 2013). Most notably, two Phase III clinical trials have recently reported on the efficacy of a silicone elastomer vaginal ring containing 25 mg dapivirine (Baeten et al., 2016; Nel et al., 2016). Dapivirine is a small-molecule experimental non-nucleoside reverse transcriptase inhibitor with nanomolar potency against HIV-1 and good safety when administered vaginally (Chen et al., 2015; Nel et al., 2009, 2014; Romano et al., 2009). Both trials demonstrated a reduction in the incidence of HIV-1 infection, by 27% in the ASPIRE study

and by 31% in The Ring Study. These lower-than-anticipated levels of efficacy have been attributed to poor adherence by participants in the clinical studies, particularly by younger women (Baeten et al., 2016; Nel et al., 2016). In post-hoc analysis of the data, >70% efficacy was reported for those women who used the rings consistently (Baeten et al., 2016; Nel et al., 2016). With application for regulatory approval to license the dapivirine ring product underway, attention is shifting to the development of next-generation vaginal rings, including combination antiretroviral and multipurpose prevention technology (MPT) products (Boyd et al., 2016; Clark et al., 2014; Malcolm et al., 2016, 2014; Murphy et al., 2016; Ugaonkar et al., 2015; Young Holt et al., 2014).

Resistance monitoring studies in Sub-Saharan Africa have reported rates of primary drug resistance mutations of up to 20% (World Health Organization, 2016). This phenomenon is an emerging trend (Gupta et al., 2012; Kumar and Pillay, 2007). As with highly active antiretroviral therapy for HIV treatment, a combination antiretroviral product might alleviate concerns over lower efficacy in settings with frequent primary drug resistance by targeting multiple mechanisms in the viral replication cycle, or through additive or synergistic effects leading to higher antiviral activity, and greater breadth of activity, against emerging resistant strains (Shattock and Rosenberg, 2012). These benefits only become more acute for large-scale introduction of a new prevention product without equivalent roll-out of means to identify and correct for resistance development. For this reason, combination HIV microbicide strategies are actively being developed (Fetherston et al., 2013a; Johnson et al., 2010; Moss et al., 2016; Srinivasan et al., 2016). Microbicide combinations comprising dapivirine – the current lead microbicide candidate – and other antiretroviral drugs having a different mechanism of action

would seem particularly appropriate. Protease inbibitors, such as darunavir, offer potential in combination microbicide strategies, since these molecules act at a later step in the replication cycle and have a relatively high barrier to the development of resistance (Herrera and Shattock, 2012; Stefanidou et al., 2012; Wensing et al., 2010). The IC_{50} of darunavir against HIV strains is typically 1–2 nM (Brower et al., 2008; Lv et al., 2015; Tie et al., 2012). Previous studies showed no toxicological effects when vaginal fluid concentrations of darunavir exceeded >20,000 the IC_{50} value (Murphy et al., 2014).

Non-human primate models have played an important role in the development of our understanding of the complex mechanisms of HIV infection and in the testing of new vaccine and vaginal microbicide strategies (Bernard-Stoecklin et al., 2014; Dezzutti, 2015; Henning et al., 2015; Hessell and Haigwood, 2015; Veazey et al., 2012). Three macaque species – the rhesus macaque (*Macaca mulatta*), the pig-tailed macaque (*Macaca nemestrina*) and the cynomolgus macaque (*Macaca fascicularis*) – are widely used in vaginal ring pharmacokinetic and viral challenge studies (Fetherston et al., 2013b; Hsu et al., 2014; Malcolm et al., 2012b; Moss et al., 2014, 2012; Murphy et al., 2014; Singer et al., 2012; Smith et al., 2017) as these species are susceptible to the closely related simian (SIV) and simian/human chimera (SHIV) immunodeficiency viruses that produce pathologies similar to that seen with HIV-1 infection in humans (Dezzutti, 2015; Pereira et al., 2012). Since there are differences in size/weight between these macaque species, vaginal rings should ideally be customised for optimal fit (Promadej-Lanier et al., 2009). However, there have been no reports to date on the impact of ring size on pharmacokinetic properties.

Here, we investigate the pharmacokinetics of a combination dapivirine-darunavir ring in cynomolgus macaques. We have previously reported that 25 × 6 mm rings were poorly positioned within the vaginal vault due its relatively large size (Murphy et al., 2014). Here, we report smaller ring devices for this species and investigate the impact of ring size and drug loading on *in vitro* and *in vivo* performance.

2. Materials and methods

2.1 Materials

Medical grade, addition-cure, silicone elastomer (DDU-4320) was supplied by Nusil Silicone Technology Inc. (Carpinteria, CA, USA). Non-micronised darunavir ethanolate (DRV) and micronised dapivirine (DPV) were kindly provided by Janssen Research and Development (Beerse, Belgium) and the International Partnership for Microbicides (Silver Spring, MD, USA), respectively. High performance liquid chromatography (HPLC) grade acetonitrile, isopropanol (IPA), methanol, dichloromethane, trifluoroacetic acid (TFA) and titanium dioxide were obtained from Sigma Aldrich (Gillingham, UK). 19-norethindrone was from Sigma Aldrich (Gillingham, UK) and LGM Pharma (Nashville, TN, USA). Dapivirine-d11 was obtained from Toronto Research Chemicals (Toronto, Canada) and darunavir-^{13}C6 was from Alsachim (Illkirch-Graffenstaden, France). Deionised water, resistivity >18 mΩ/cm^2, was obtained from a Millipore Direct-Q 3 UV Ultrapure water system (Watford, UK). Simulated vaginal fluid (SVF, pH 4.2) was prepared using analytical grade reagents according to a method described previously (Owen and Katz, 1999). Briefly, for 1 L of SVF, 3.51 g of NaCl, 1.40 g KOH, 0.222 g of $Ca(OH)_2$, 0.018 g of bovine serum albumin, 2 g lactic acid, 1 g acetic acid, 0.16 g glycerol, 0.4 g

urea and 5 g glucose were dissolved in ~ 900 mL of deionised water, the pH adjusted to 4.2 with concentrated HCl and the solution adjusted to volume with deionised water.

2.2 Ring manufacture

Matrix-type, silicone elastomer vaginal rings with three different geometries (25.0 × 6.0 mm, 20.0 × 4.5 mm and 15 × 3.0 mm; external and cross-sectional diameters, respectively) were manufactured by reaction injection molding using a laboratory-scale ring manufacturing machine fitted with custom aluminium molds (Figure 1a). Each ring contained 5.81% w/w dapivirine and 17.44% w/w darunavir, equivalent to 100 mg dapivirine and 300 mg darunavir per ring for the 25 × 6 mm ring and proportionately lower quantities for the two smaller rings (Table 1). For batch manufacture of each ring, the required quantities of dapivirine and darunavir were added to both parts A and B of the silicone elastomer base in polypropylene containers. After mixing (2000 rpm, 5 min, SpeedMixer DAC 150 FVZ-K, Polymer Systems, UK), the containers were degassed in a vacuum chamber (0.143 Torr for 30 min; LACO Technology, Salt Lake City, UT, USA), and then stored sealed at 4 °C for 60 min. Parts A and B of the silicone/drug mixture were combined in a 1:1 ratio and mixed (3000 rpm for 30 s) before being injected into pre-heated (80 °C) vaginal ring molds and cured for 3 min. The nominal drug loadings and mean weights for each ring size are presented in Table 1.

2.3 In vitro release testing

In vitro release testing of the rings was conducted over 28 days using both a 1:1 mixture of isopropanol and water (IPA/H_2O) and SVF. Both media have been widely used for *in vitro* release testing of vaginal rings (Boyd et al., 2016; Fetherston et al., 2013b; Malcolm et al., 2016;

McCoy et al., 2017; Murphy et al., 2016; Murphy et al., 2014). Isopropanol/water has been used extensively for *in vitro* release testing throughout product development of the dapivirine ring, primarly due to its ability to provide sink conditions for the very poorly-water soluble dapivirine and its discriminatory power for the key critical quality attributes of the drug product (McCoy et al., 2017; Woolfson et al., 2010). Similar high isopropanol content buffer mixtures are routinely used for USP compendial dissolution testing of oral tablets containing the poorly water soluble anti-malarial drug atovaquone, and a medium containing 5% methanol is also recommended for the injectable suspension of triptorelin pamoate (U.S. Food and Drug Administration, 2018; https://www.accessdata.fda.gov/scripts/cder/dissolution/ (accessed 06/01/18)). SVF is intended to simulate the chemical composition of vaginal fluid, with a particular emphasis on modelling pH and osmolarity; it is not intended to mimic the viscosity of vaginal fluid (Owen and Katz, 1999). However, although SVF is considered more physiologically relevant, its low solvating power creates many challenges in its use as an *in vitro* release medium for poorly water-soluble drugs like dapivirine (McCoy et al., 2017). In this study, we have performed *in vitro* release testing using both IPA/water and SVF media so as to explore both sink and non-sink condition models.

Rings (n=4) were individually placed in 250 mL glass flasks (Duran® GLS 80®) containing 100 mL of the either IPA/H_2O or SVF. This relatively large volume of release medium is routinely used for *in vitro* release testing of vaginal rings, despite much lower volumes of vaginal fluid – typically < 1 mL – measured *in vivo* (Owen and Katz, 1999). The larger volume allows for complete coverage of the entire ring in the flask. Sealed flasks were placed in an orbital shaking incubator (Infors HT Unitron, Switzerland; 37 °C, 60 rpm, throw 25 mm). After 24 h (± 15 min),

each flask was removed from the incubator and a 2 mL sample of release medium removed for HPLC analysis. The remaining release medium was discarded and replaced with a fresh 50 mL volume. Sampling was conducted daily except for weekends. The daily release medium volume used was 50 mL with the exception of day 0 and weekends when the volume of release medium added was doubled to maintain sink conditions. The quantities of dapivirine and darunavir released were quantified by HPLC, as described previously (Murphy et al., 2014). Briefly, 10 µL of each sample was injected onto a Luna 5 µm C18 column (150 × 4.6 mm; Phenomenex, UK) and run at a flow rate of 1 mL/min in a mobile phase of 45% 0.1% TFA in water, 55% methanol with the column held at 30˚C. Both compounds were detected at 257 nm with approximate run times of 10.5 and 12 min for darunavir and dapivirine respectively. Daily and weekend release values were calculated and used to plot daily and cumulative release versus time graphs.

2.4 Size fitting and pharmacokinetic study in macaques

A size fitting and pharmacokinetic study in adult female Mauritian cynomolgus macaques (*Macaca fascicularis*, weight range 3–7 kg) was performed at Commissariat à l'Energie Atomique (CEA), IDMIT infrastructure, Fontenay-aux-Roses, France in accordance with French national regulations and under the supervision of national veterinary inspectors (CEA Permit Number C 92-032-02). All experimental procedures were carried out in strict accordance with European guidelines for non-human primate care (EU Directive 2010/63/EU) for protection of animals used in experimentation and other scientific purposes and the Weatherall Report. The CEA also complies with the Standards for Humane Care and Use of Laboratory Animals, of the Office for Laboratory Animal Welfare (OLAW, USA). The study was reviewed and approved by the EU FP7 Combined Highly Active Antiretroviral Microbicide (CHAARM) program. The

study and procedures were approved by ethics committee "Comité Régional d'Ethique pour l'Expérimentation Animale Ile-De-France Sud" with notification number 10-062. All efforts were made to minimize suffering, including improved housing conditions with enrichment opportunities (e.g. 12 hr / 12 hr light-dark scheduling, provision of treats, constant access to water supply, and regular play interaction with staff caregivers and research staff). Experimental procedures were performed while animals were under sedation with 10 mg/kg (body weight) of ketamine hydrochloride.

In the ring size fit study, two animals were used per ring size. Drug-free silicone elastomer vaginal rings measuring 20 × 4.5 mm or 15 × 3 mm (external diameter × internal diameter) were atraumatically inserted into the macaque vagina and worn continuously over 28 days. Vaginal cytology was examined using cervico-vaginal lavage (CVL) samples taken at baseline and after 1, 7, 14 and 25 days. Blood samples were taken periodically to determine the blood cell formula. Colposcopy was performed one day before and one day after ring administration, and on days 7, 14 and 25 to determine ring position and check for visual signs of inflammation. Progesterone levels were measured weekly throughout the experiment to determine the stage of the menstrual cycle (Figure S1. Supplementary Information). Animals were necropsied on day 28 and histological examination of vaginal tissue was performed. A summary of the sample collection schedule is presented in Table 2(a).

For the macaque pharmacokinetic study, vaginal rings (20 × 4.5 mm; n=4) nominally containing 47 mg dapivirine and 142 mg darunavir were tested in four macaques over 28 days. Vaginal fluids were collected using Weck-Cel® sponges (Beaver Visitec International) at baseline and

after 1, 7, 14 and 28 days, as described previously (Murphy et al., 2014). Briefly, pre-weighed Weck-Cel sponges were placed in the vaginal vault for 2 min to absorb fluid. Upon removal, sponges were reweighed to calculate the collected vaginal fluid weight and stored at –80°C until further analysis. Blood was sampled at baseline and after 1, 7, 14, and 28 days for dapivirine and darunavir quantification and to measure the level of progesterone in serum. Progesterone concentrations were quantified using IBL progesterone ELISA kit (RE52231, IBL International, Germany) to determine the phase of menstrual cycle during ring placement. Animals were necropsied on day 28 and histological changes in the vaginal tissue were monitored to document any signs of inflammation or mucosal damage. Vaginal fluid and serum samples were shipped on dry ice to King's College London for quantification of dapivirine and darunavir by HPLC-MS/MS. A summary of the sample collection schedule for the pharmacokinetic study is presented in Table 2(b).

2.5 Quantification of dapivirine and darunavir in vaginal fluid and serum

Dapivirine and darunavir were quantified in macaque vaginal fluid and serum by HPLC-MS/MS using an Agilent 1100 LC system coupled to a Waters Quattro LC mass spectrometer for vaginal fluid samples and a Thermo Scientific Accela Pump and Autosampler coupled to a Thermo Scientific TSQ Quantum Access mass spectrometer for serum samples. Dapivirine-d11 and darunavir-^{13}C6 were used as internal standards. Full details are available in the Supplementary Information accompanying this manuscript.

2.6 Pre- and post-use content of dapivirine and darunavir rings

Initial and residual dapivirine and darunavir contents in rings were quantified using a solvent extraction method after manufacture and after completion of both *in vitro* release and *in vivo* pharmacokinetic testing in macaques (n=4). Individual rings were sectioned into ~2 mm pieces and placed into a 250 mL glass flask. 5 mL of a 1 mg/mL solution of 19-norethindrone in methanol was added to each flask, followed by 95 mL of dichloromethane. Flasks were sealed and placed in an orbital shaking incubator (Infors HT Unitron, Switzerland; 37 °C, 60 rpm, throw 25 mm). After 72 hr, the flasks were removed, cooled to room temperature, and a 2 mL aliquot transferred to a boiling tube and evaporated to dryness. The residue was reconstituted in 10 mL methanol with vortex mixing for 30 s and sonication for 40 min. A further 1:10 dilution of the sample in methanol was performed prior to HPLC analysis. 10 µL of each sample was injected onto a Luna 5 µm C18 column (150 × 4.6 mm) held at 30˚C and run using a mobile phase of 25% water, 75% methanol with a flow rate of 0.75 mL/min. Dapivirine, darunavir and norethindrone were all detected at 257 nm with retention times of 9.5, 3.5 and 5.1 minutes, respectively.

2.7 Statistical analyses

Mean maximum serum or vaginal fluid concentrations (C_{max}) and the area under the curve up to the last measured concentration (AUC_{last}) for both DPV and DRV were calculated and compared with previously published values using the Mann Whitney test (Murphy et al., 2014).

3. Results and Discussion

3.1 Ring design and manufacturing considerations

The amount of drug that could practically be loaded into the smaller ring sizes was constrained by the volumes of the rings (Table 1). For this reason, it was not possible to produce smaller rings with the same total drug loadings as reported previously for the 25 × 6 mm ring (i.e. 100 mg DPV, 300 mg DRV). Instead, we manufactured rings having an equivalent % w/w drug concentration to those used previously. The size, mean weight and nominal drug loadings for each size of ring are presented in Table 1 alongside mean measured assay (content) values. The measured drug contents were all within ±10% of the nominal value for both DPV and DRV. These data indicate that the manufacturing method and newly designed molds used for the 20 × 4.5 and 15 × 3 mm rings are capable of producing rings to the desired specification and that it is possible to accurately and reproducibly manufacture smaller-sized silicone elastomer rings with greater than 20% w/w drug content.

3.2 Ring size fitting in cynomolgus macaques

Colposcopic images of the rings *in situ* clearly showed the rings situated either just above or around the cervix (Figure 1b). No cervical or vaginal lesions were observed with the 20 × 4.5 mm ring. However, the 15 × 3 mm rings entrapped the cervix and caused vaginal erosions. Histological analyses of the vaginal tissues collected after 28-day use showed that both tissue samples had a similar lesional profile including some epithelial lesions of variable severity and distribution. These lesions, even when focally severe, could not be correlated unambiguously to the presence of a vaginal ring and the size of the ring did not seem to impact the severity of the lesions. On the basis of these results, the 20 × 4.5 mm ring was deemed most suitable for this species of macaque. Vaginal erythematous areas have been observed previously with use of a contraceptive, levonorgestrel-releasing, silicone elastomer vaginal ring in women (Bounds et al.,

1993). In that study, it was not possible to conclusively determine if the erythemas were caused by hormonal or physicomechanical effects associated with the ring device.

3.3 In vitro release of dapivirine and darunavir

Daily and cumulative *in vitro* release vs. time profiles for DPV and DRV from combination rings of different sizes are presented in Figure 2 (a–d) for release into IPA/H_2O and Figure 3 (a–d) for release into SVF (cumulative % release plots are presented in Figure S2. Supplementary Information). Consistent with permeation-controlled release from a non-biodegradable matrix-type device, both drugs displayed high initial release into IPA/H_2O with declining rates thereafter (Figure 2a and 2b). This trend is blunted to some extent in SVF due to its poorer solvating power (Figure 3a and 3b). A summary of the release rates with confidence intervals, determined from the gradients of the best-fit lines, and coefficients of determination for each ring size and release media are presented in Table 3.

The IPA/H_2O release medium was able to discriminate between the increasing amounts of DPV released as a function of increased ring size and initial DPV loading. DPV release was effectively modelled by a root-time model (Figure 2c) with coefficients of determination >0.999 (Table 3) (Higuchi, 1963). The cumulative release plots of DRV are not as well described by the root-time model (Figure 2d), with the coefficients of determination for the intermediate and smaller ring size <0.98 indicating deviation from root-time kinetics (Table 3). As expected, the release rates for both compounds into SVF are much lower than into IPA/H_2O (Table 3). In particular, DPV release is substantially reduced and the cumulative release vs. time profile is linear (Figure 3). Surprisingly, the total amount of DRV released into both release media is greater for the 20 × 4.5 mm ring compared to the 25 × 6 mm ring, despite a higher overall drug

content in the larger ring and an increase in surface area available for release (Figure 2d, 3d and Table 1). Darunavir ethanolate has previously been shown to be stable at the temperature of ring manufacturing (80˚C) (Gyseghem et al., 2009; Murphy et al., 2014). However, the ethanolate converts to the hydrate in environments of moderate or high relative humidity. Conversion to an amorphous form can also occur on heating of either solvate, but occurs at a lower temperature with the hydrate (Gyseghem et al., 2009). Differential scanning calorimetry (DSC) analysis of the supplied DRV showed that conversion of the ethanolate to the hydrate has occurred (Figure S3. Supplementary Information,). While the ethanolate is largely stable upon heating up to about 80˚C, the hydrate shows maximal weight loss around 75˚C (Gyseghem et al., 2009). Therefore, during ring manufacture there is potential for formation of amorphous DRV within the ring. Ring sections analysed by DSC showed that the 20 × 4.5 mm ring exhibited a relatively small DRV melting peak area compared to the other two ring sizes, for an equivalent sample weight (Table S1. Supplementary information), confirming a greater proportion of amorphous DRV. This explains the increased release rates for 20 × 4.5 mm ring relative to the larger 25 × 6 mm ring, and is likely attributed to the higher rate of dissolution of amorphous DRV compared with the crystalline ethanolate or hydrate forms. It is not yet clear why this particular ring size would lend itself to increased formation of amorphous material. However, this finding serves to underline the impact of salt or crystal form stability and any interconversions that may take place. It also highlights the importance of ensuring stability under the processing conditions used in ring manufacture, as well as on storage.

3.4 Pharmacokinetics and residual ring content of dapivirine and darunavir

A pharmacokinetic study in cynomolgus macaques was conduced to determine whether drug release and biodistribution with the 20 × 4.5 mm rings were significantly different from the 25 × 6 mm rings. Concentration vs. time plots for DPV and DRV in both serum and vaginal fluid are presented in Figure 4. Statistical pharmacokinetic parameters are presented in Table 4. Note that some samples originally collected with the 25 × 6 mm rings (at 8 hr and 2 days) were not collected for the 20 × 4.5 mm rings. In particular, the maximum concentrations in vaginal fluid (C_{max}) should be interpreted with caution as they were measured at the 8 hr time point for the 25 × 6 mm ring. However, both studies were conducted by the same laboratory and all other study details were kept constant between the two studies to allow for direct comparison. Mean maximum serum concentrations for DPV and DRV were 673 and 1046 pg/mL, respectively. Surprisingly, the serum levels of both drugs are similar, or even slightly higher, with the 20 × 4.5 mm ring compared to the 25 × 6 mm ring (Figure 4a and 4b). This is despite the greater ring surface area and total amount of drug loaded in the larger ring. Mean C_{max} values for DPV and DRV in serum were not significantly different when assessed using the Mann-Whitney test ($p=0.1143$ and 0.0571 for DPV and DRV respectively). Mean AUC_{last} values for DRV were significantly higher in the 20 × 4.5 mm ring group $p=0.0286$, while DPV values were not significantly different, $p=0.2$. This increase in DRV release may be related to the unexpected production of amorphous drug, with potentially substantially enhanced release, during ring manufacture. Comparing vaginal fluid levels (Figure 4c and 4d) also showed no significant difference between C_{max} or AUC_{last} values between the two ring sizes for DPV or DRV ($p=0.6857$ and 0.8857 for DPV and $p=0.1143$ and 0.3429 for DRV, respectively).

Quantification of residual drug content of vaginal rings following *in vitro* release and *in vivo* testing has previously been used to gain insight into the total amount of DPV and DRV released and as a surrogate measure of user adherence to rings in clinical trials (Baeten et al., 2016; Murphy et al., 2014; Nel et al., 2016, 2009; Spence et al., 2016). Here, we measured residual drug content in rings following *in vitro* release testing and following return of used rings from the PK study. The results, presented in Table 5, demonstrate that the residual content assay can easily differentiate between rings release tested in IPA/H_2O and SVF. In all but one case, the measured residual content combined with the mean cumulative release value summed to give a content value within 5% of the nominal drug loading. The measured initial and residual content values of rings used in macaques indicated low overall quantities of drug release for both DPV or DRV (Table 5). Previous studies testing the 25 mg DPV ring in sheep and humans indicate release of ~4 mg over 28 days (Holt et al., 2015; Spence et al., 2016). The apparent disparities between the initial and final content, seen for example with the measured initial and final DRV levels of rings used in the PK study, highlight the difficulties associated with this approach where there is a high initial drug loading and only a very small fraction of the drug released during testing. In addition, as content testing is destructive, only mean values for the level of ring content can be determined prior to ring use. Thus, inherent manufacturing variability as well as analytical method limitations and experimental error may serve to cloud the results. We have previously attempted to correlate DPV vaginal fluid and plasma concentrations with the quantity of DPV released during *in vitro* testing (Malcolm et al., 2016). Here, comparisons are more difficult since intial loadings were different between the rings. However, in line with previous work, we observed slightly lower, but broadly similar vaginal fluid and similar plasma concentrations for DPV. Surprisingly, the DRV levels appear to be slightly higher with the

smaller ring, and, although the differences are generally not statistically significant, they are in line with the trends seen *in vitro* into both IPA/H_2O and SVF. As discussed previously, this is likely due to formation of amorphous DRV during ring manufacture. Overall, vaginal fluid and serum concentrations of DPV and DRV in macaques following administration of 20 × 4.5 mm rings were not significantly different to those observed with 25 × 6 mm rings, despite the decreased surface area available for release and the lower total amount of drug present in the smaller rings (Table 1). This may reflect the better positioning of the smaller ring within the vagina and serves to emphasize the importance of appropriate ring placement when conducting pharmacokinetic studies in this species of macaque. A contribution to the DRV release may also be due to the potential production of amorphous drug during ring manufacture. These data suggest that ring geometry and placement plays an important role in the evaluation of the pharmacokinetics of potential microbicides in macaques.

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None to declare.

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Table 1. Ring geometries, mean weights, and nominal and measured drug loadings (in mg per ring) of rings containing 5.81% w/w DPV and 17.44% w/w DRV. Mean and SD of 4 replicates.

External × internal diameter (mm)	Calculated surface area and volume (cm^2/cm^3)	Mean weight (g) ± SD	Nominal DPV loading (mg)	Mean measured DPV content (mg) ± SD	Nominal DRV loading (mg)	Mean measured DRV content (mg) ± SD
25 × 6	1125, 1688	1.797 ± 0.003	100	103.1 ± 1.8	300	303.0 ± 11.9
20 × 4.5	668, 774	0.802 ± 0.002	47	48.9 ± 0.7	142	145.7 ± 4.9
15 × 15	355, 266	0.287 ± 0.003	17	18.2 ± 0.4	50	54.5 ± 0.9

DPV – dapivirine, DRV – darunavir, SD – standard deviation

Table 2. Time points for collection of biological samples from macaques during period of blank ring placement and during pharmacokinetic study.

Sampling procedure	Time after ring insertion (days)								
	-7	-1	0	1	7	14	21	25	28
A. During placement of blank ring									
Vaginal fluid for cytologic analysis		x		x	x	x		x	
Colposcopy		x		x	x	x		x	
Blood for progesterone quantification	x	x			x	x	x	x	
Tissue for histological evaluation									x
B. During pharmacokinetic study									
Vaginal fluid for drug quantification			x	x	x	x		x	
Blood for drug quantification in serum			x	x	x	x		x	
Blood for progesterone quantification*	x		x		x	x	x	x	x
Tissue for histological evaluation								x	

* Day -7 progesterone levels recorded in 3 of 4 macaques

Table 3. Summary *in vitro* release data for dapivirine (DPV) and darunavir (DRV) from silicone elastomer ring formulations into 1:1 mixture of isopropanol and water (IPA/H_2O) and simulated vaginal fluid (SVF) release media. Ring loadings are as detailed in Table 1. Release rates are the gradients obtained from linear regression analysis of the mean cumulative release vs. root time or time plots (Figures 2c, 2d, 3c and 3d). r^2 values, representing the coefficient of determination, are also reported for the plots.

Ring size (ED × ID, mm)[#]	Drug	Release medium	Release rate (95% CI) (μg/day$^{0.5}$) [a](μg/day)	r^2 value
25 × 6	DPV	IPA/H_2O	4435 (4413–4458)	0.9999
20 × 4.5	DPV	IPA/H_2O	2791 (2765–2816)	0.9997
15 × 3	DPV	IPA/H_2O	1372 (1354–1390)	0.9993
25 × 6	DRV	IPA/H_2O	1190 (1163–1218)	0.9978
20 × 4.5	DRV	IPA/H_2O	1403 (1299–1507)	0.9781
15 × 3	DRV	IPA/H_2O	527 (479.9–574.1)	0.9685
25 × 6[a]	DPV	SVF	43.09 (41.9–44.3)[a]	0.9969
20 × 4.5[a]	DPV	SVF	37.70 (36.5–38.9)[a]	0.9958
15 × 3[a]	DPV	SVF	29.59 (28.4–30.8)[a]	0.9945
25 × 6	DRV	SVF	456.5 (449.9–463.1)	0.9991
20 × 4.5	DRV	SVF	492.7 (489.5–495.9)	0.9998
15 × 3	DRV	SVF	277.6 (276.0–279.2)	0.9999

[#] ED – external diameter, ID – internal diameter

[a] DPV release into SVF is better described by a plot of cumulative release against time (μg/day) i.e. zero order release, possibly representing solubility controlled release

Table 4. Statistical pharmacokinetic parameters for dapivirine (DPV) and darunavir (DRV) in macaque serum and vaginal fluid following use of 25 × 6 mm and 20 × 4.5 mm vaginal rings for 28 days. Values for 25 × 6 mm rings used with permission (Murphy et al., 2014).

Drug	Ring size (ED × ID, mm)	Vaginal fluid			Serum		
		C_{max}[a] (ng/g)	T_{max}[a, b] (h)	*AUC* (μg·h/g)	C_{max}[a] (pg/mL)	T_{max}[a, b, c] (h)	AUC (pg·h/mL)
DPV	25 × 6	19260 ± 5675	8	6336 ± 3999	206 ± 108	108[b]	100945 ± 51871
DPV	20 × 4.5	11507 ± 3269	24	4206 ± 689	673 ± 557	336	241017 ± 165897
DRV	25 × 6	69916 ± 11798	8	15863 ± 11401	178 ± 116	24	43009 ± 16236
DRV	20 × 4.5	110441 ± 66089	24	22585 ± 5385	1046 ± 1058	24/336[b]	238740 ± 181639

ED – external diameter, ID – internal diameter

[a] Note that whilst both studies collected samples at 1, 7, 14 and 28 days, the sampling schedule for the study involving 25 × 6 mm rings also had time points at 8 hr and 2 days which were not collected for the 20 × 4.5 mm rings.[26]

[b] The most common time point reporting the maximum concentration.

[c] No time was most common; value reported is either the mean of two middle times or both times are reported individually.

Table 5. Mean ring content values (n=4 in each case) after 28 days use in an *in vitro* release test compared with initial content values assessed on rings that were not tested and similar sized rings that were used in macaques for 28 days (*in italics*).

Ring size (ED × ID, mm)	Drug and theoretical loading (mg per ring)	Mean initial content (mg) ± SD	Mean cumulative release (mg)		Mean residual content (mg) ± SD		Total recovered content (mg)	
			IPA/H_2O	SVF	IPA/H_2O	SVF	IPA/H_2O	SVF
25 × 6	DPV 100	103.1 ± 1.8	22.8	1.2	77.4 ± 2.6	99.2 ± 3.8	100.2	100.4
	DRV 300	303.0 ± 11.9	6.6	2.2	290.2 ± 10.0	297.1 ± 11.5	296.8	299.2
20 × 4.5	DPV 47	48.9 ± 0.7	8.6	1.1	34.3 ± 0.4	47.0 ± 0.9	42.7	48.0
	DRV 142	145.7 ± 4.9	8.6	2.4	139.3 ± 1.7	143.4 ± 3.5	147.7	145.8
15 × 3	DPV 17	18.2 ± 0.4	7.2	0.8	10.9 ± 0.1	17.2 ± 0.1	18.10	18.0
	DRV 50	54.5 ± 0.9	3.8	1.4	48.2 ± 0.6	50.8 ± 1.5	52.0	52.2
20 × 4.5	*DPV 47*	*50.3 ± 1.4*	*N/A*		*50.1 ± 0.8*		*N/A*	
	DRV 142	*155.9 ± 3.6*	*N/A*		*158.3 ± 4.9*		*N/A*	

DPV – dapivirine, DRV – darunavir, SD – standard deviation, IPA/H_2O – a 1:1 mixture of isopropanol and water, SVF – simulated vaginal fluid.

Values for 25 × 6 mm rings used with permission (Murphy et al., 2014).

List of Figure Legends

Figure 1. (a) Mold design for fabrication of 20 × 4.5 mm and 15 × 3.0 mm rings. Blank (non-medicated) silicone elastomer rings are shown on the right hand side; rings on the left hand side contain 5% w/w titanium dioxide. (b) Blank rings *in situ* examined by colposcopy; left – 20 × 4.5 mm ring, right – 15 × 3.0 mm ring; A – cervix, B – vagina, C – ring.

Figure 2. Daily *in vitro* release versus time (a and b) and cumulative *in vitro* release versus root time (c and d) profiles for dapivirine (DPV) and darunavir (DRV) into 50 mL of a 1:1 mixture of isopropanol and water over 28 days from combination rings of different geometries each containing 5.81% DPV and 17.44% DRV (w/w). Each value is the mean of four replicates and error bars (often smaller than the plot symbols) in the daily release vs. time profiles denote standard deviations.

Figure 3. Daily *in vitro* release versus time (a and b) and cumulative *in vitro* release versus (c) time and (d) root time profiles for dapivirine (DPV) and darunavir (DRV) into 50 mL simulated vaginal fluid over 28 days from combination rings of different geometries each containing 5.81% DPV and 17.44% DRV (w/w). Each value is the mean of four replicates and error bars in the daily release vs. time profiles denote standard deviations.

Figure 4. Serum (a and b) and vaginal fluid (c and d) concentration versus time profiles for dapivirine (DPV) and darunavir (DRV) during 28 day placement in cynomolgus macaques of silicone elastomer matrix-type rings either 25 × 6 mm or 20 × 4.5 mm in size, each containing 5.81% w/w DPV and 17.44% w/w DRV. Each value is the mean of four replicates and error bars denote standard deviation. One day 7 and day 28 serum time point is missing for one macaque, and one day 14 serum time point is missing for another. Data for 25 × 6 mm rings used with permission (Murphy et al., 2014).

Figure S1. Measured progesterone levels for each macaque throughout the 28 day PK study.

Figure S2. Cumulative % release graphs (a) dapivirine cumulative % release into IPA/H_2O v root time, (b) dapivirine cumulative % release into SVF v time, (c) darunavir cumulative % release into IPA/H_2O v root time, (d) darunavir cumulative % release into SVF v root time.

Figure S3. DSC trace of darunavir ethanolate (after long-term storage in an uncontrolled humidity environment) showing substantial conversion to the hydrate.

Supplementary Information

Quantification of dapivirine and darunavir in vaginal fluid and serum

Dapivirine and darunavir were quantified in macaque vaginal fluid and serum by HPLC-MS/MS using an Agilent 1100 LC system coupled to a Waters Quattro LC mass spectrometer for vaginal fluid samples and a Thermo Scientific Accela Pump and Autosampler coupled to a Thermo Scientific TSQ Quantum Access mass spectrometer for serum samples. Dapivirine-d11 and darunavir-^{13}C6 were used as internal standards.

Vaginal fluid samples

Weck-Cel Spears were extracted with acetonitrile. Extracts (100 µL) were combined with both internal standards and diluted 10 times with 0.1% formic acid in 50% acetonitrile and transferred to injection vials. The calibration ranges for the vaginal fluid assay, using a 10 µL injection volume, were 20–3000 ng and 20–10000 ng for dapivirine and darunavir, respectively. The drugs were chromatographically separated using a Thermo Hypersil Gold aQ, 50 × 2.1 mm, 3 µm column with a reversed phase gradient over 10 min. Initial conditions consisted of mobile phase A (5 mM NH_4HCO_2 in water) and mobile phase B (5 mM NH_4HCO_2 in 95% acetonitrile) at 70/30 (v/v) with a 40 °C column temperature and a 0.2 mL/min flow rate. Gradient conditions were: 0–0.2 min 30% B, 0.2–1.0 min 30–90% B, 1.0–3.0 min 90% B, 3.0–3.2 min 90–30% B, 3.2-10.0 min 30% B for re-equilibration. Under these conditions, darunavir and dapivirine displayed retention times of approximately 5.9 and 6.9 min, respectively. The mass spectrometer was operated in positive ion electrospray MRM mode. Ion source parameters were as follows: capillary voltage 3 kV, source block temperature 90°C, desolvation temperature 350°C, nebuliser gas flow 80 L/hour and desolvation gas flow 550 L/hr. The analytes and their corresponding internal standard product ions

were monitored using collision energy (eV) set at 35 and 15 for dapivirine and darunavir, respectively. Monitored MRM transitions were m/z 330.3→158.0, 330.3→170.0, 341.3→168.0, 548.3→392.0, 548.3→436.0, and 554.3→398.0 for dapivirine, dapivirine-d11, darunavir and darunavir-^{13}C6, respectively. Each transition was monitored with a dwell time of 0.25 s.

Serum samples

Serum samples, after adding internal standards and adjusting pH with 1M Ammonia solution, were extracted with methyl tert-butyl ether. Organic layers were evaporated to dryness under nitrogen at 50ºC and reconstituted in 0.1% formic acid in 70% acetonitrile. Samples were transferred to injection vials and 20 µL aliquots were injected into the LCMSMS system. The calibration ranges for the serum assay were 0.01–10 ng/mL for both dapivirine and darunavir. Dapivirine and darunavir were chromatographically separated using a Thermo Hypersil Gold aQ, 150 × 2.1 mm, 3 µm column with a reversed phase gradient over a 8 min run time. Initial conditions consisted of mobile phase A (5 mM NH_4HCO_2 in water) and mobile phase B (5 mM NH_4HCO_2 in 95% acetonitrile) at 30/70 (v/v) with a column temperature of 40 °C and a flow rate of 0.2 mL/min. The gradient conditions ramped from 70% B to 90% B between 0.2 and 2.0 min, then maintained up to 4.0 min, 90–70% B between 4.0 and 4.2 min and then maintained at 70% B up to 8 min for re-equilibration. Under these conditions darunavir and dapivirine displayed retention times of approximately 2.6 and 4.0 min, resepectively. The mass spectrometer was operated in positive ion electrospray SRM mode. Ion source parameters were as follows: Spray Voltage 3.5 kV, capillary temperature 360°C, sheath gas flow 50 Arb., auxiliary gas flow 10 Arb. The analytes and their corresponding internal standard product ions were monitored using collision energy (Arb) set at 36 and 15 for dapivirine and darunavir, respectively. Monitored MRM transitions were m/z

330.09→158.0, 330.09→170.0, 341.10→168.00, 548.20→392.06, 548.20→436.00, and 554.20→398.00 for dapivirine, dapivirine-d11, darunavir and darunavir-^{13}C6, respectively. Each transition was monitored with a scan time of 0.15 s.

Table S1. Darunavir melt peak area for samples from rings manufactured at 80˚C

Ring size (ED × ID) mm	Weight (mg)	DRV melt peak area (J/g)
15 × 3	14.65	4.577
20 × 4.5	17.15	1.975
25 × 6	14.29	3.0299

Figure 1

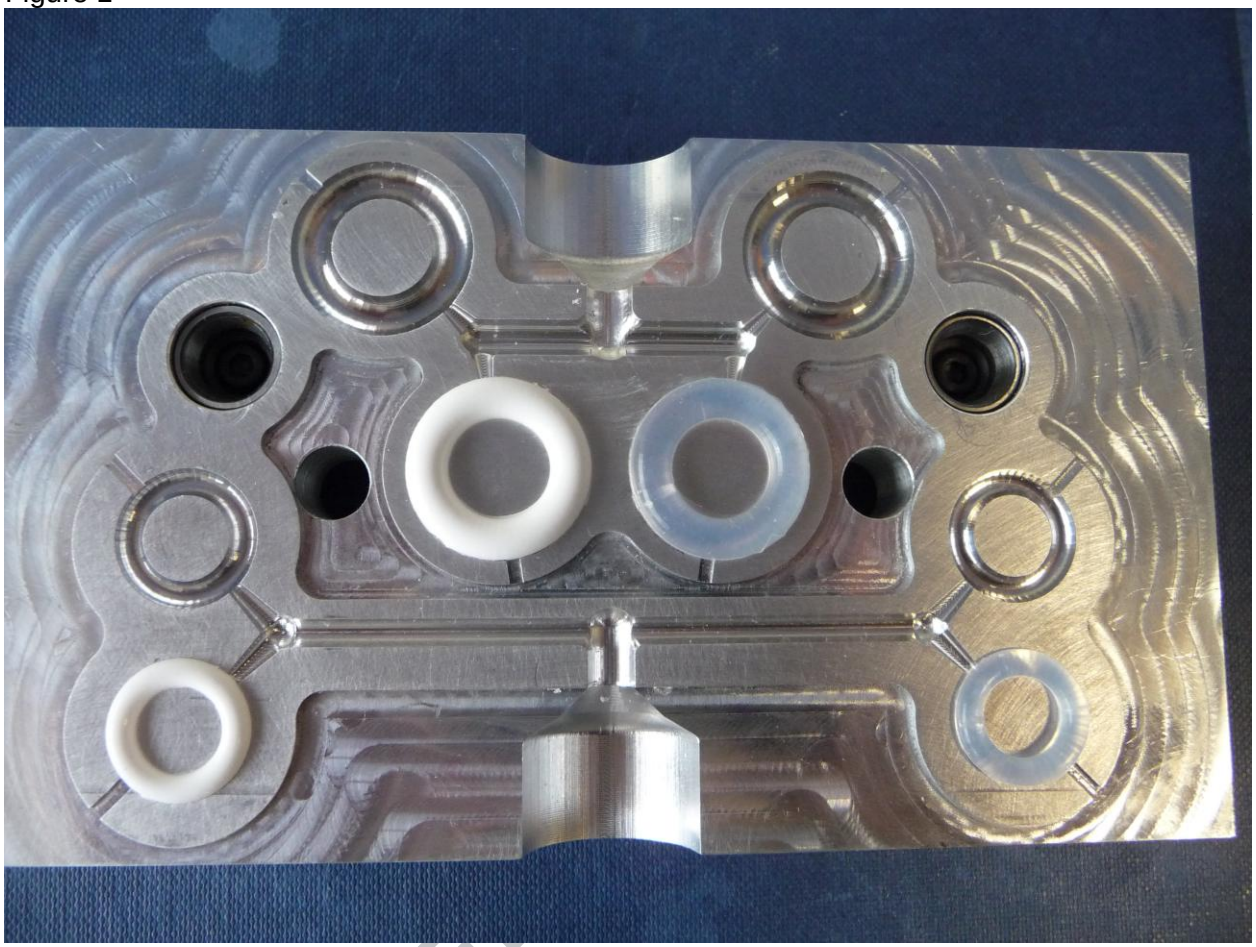
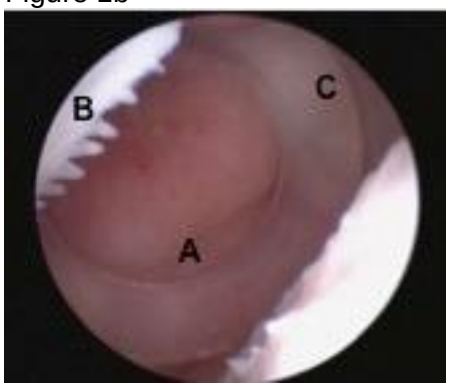


Figure 1b



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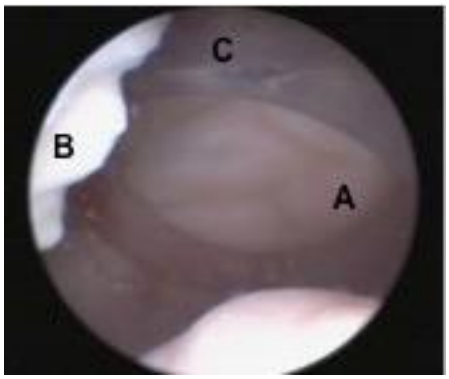

Figure 2

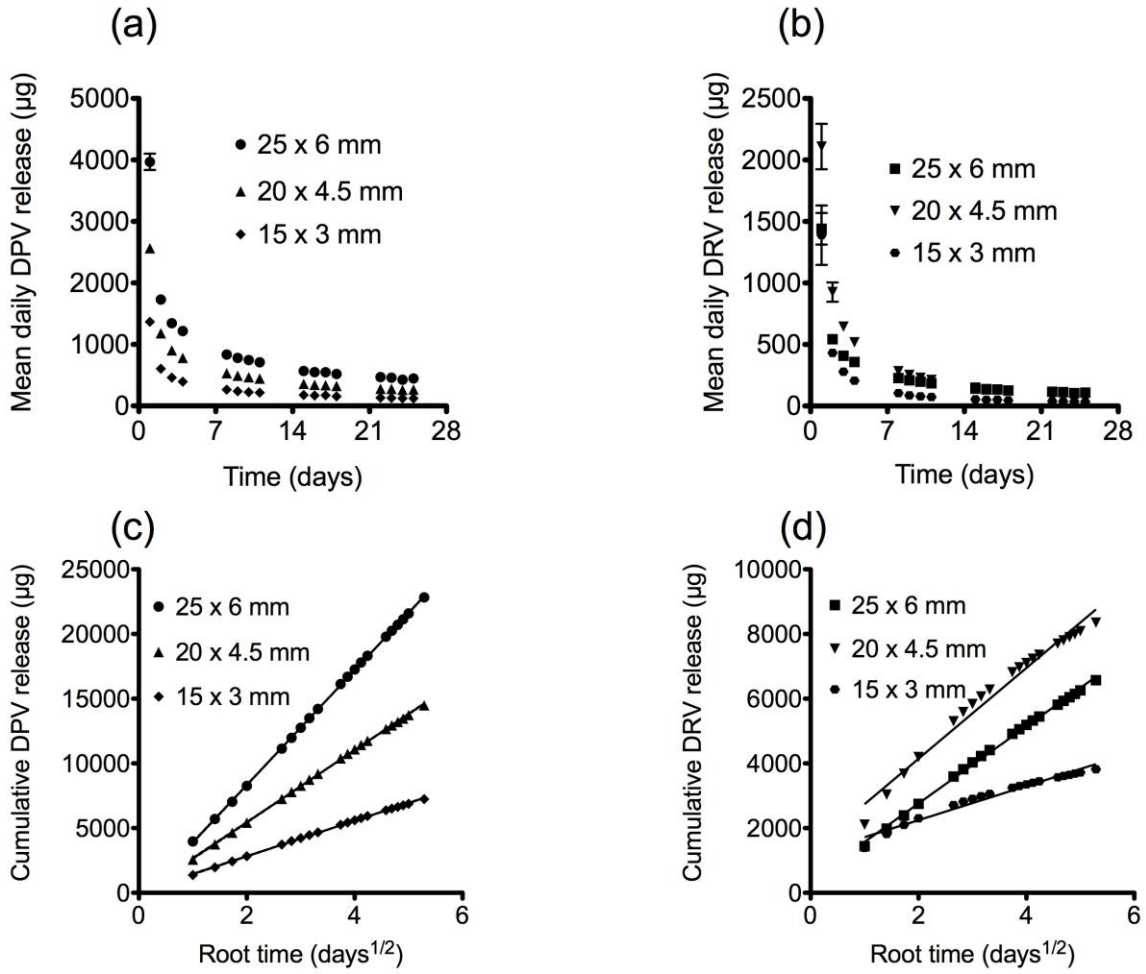

Figure 3

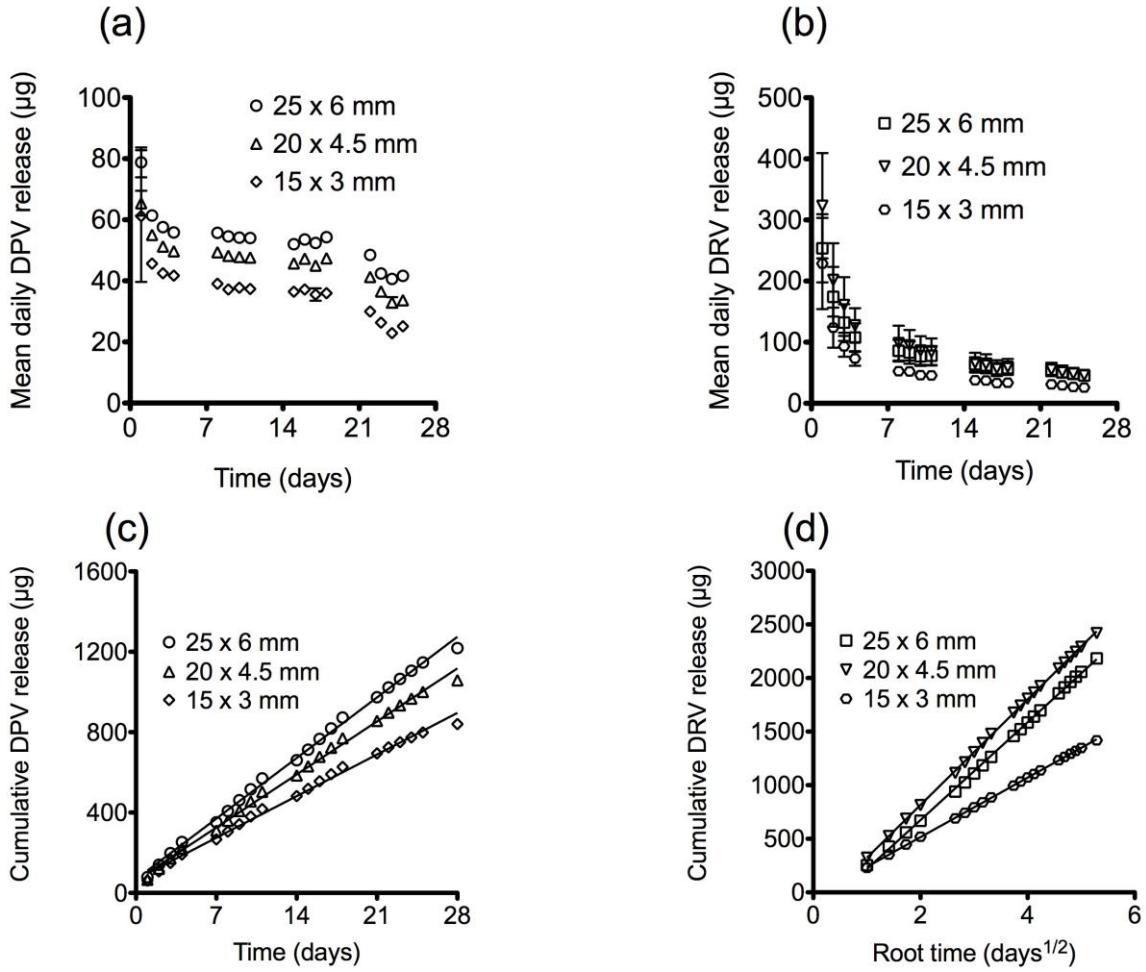

Figure 4