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In situ-generated volumetric dried plasma spots for the analysis of edaravone and metabolites in animal models

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ABSTRACT

In this study, for the first time rat plasma microsampling was carried out by means of in situ-generated volumetric dried plasma spot (vDPS) technology and applied to the determination of the neuroprotective agent edaravone and its sulphate and glucuronide metabolites. Sampling was performed using Telimmune® plasma separation cards (vPSC), which allow the formation of volumetrically accurate dried plasma spots (3 μL) from blood drops deposited on them. After accelerated forced drying and solvent extraction in methanol, drying and redissolution, analytes were baseline separated and quantified through an original HPLC-MS/MS analytical method. Validation assays provided excellent results, with detection limits between 0.7 and 1.7 ng/mL, and quantitation limits between 2.0 and 5.0 ng/mL. Extraction yields were higher than 81 % and precision was lower than 14.1 % (relative standard deviation, RSD). The volumetric microsampling approach offers a much less invasive and stressful sampling. The vPSC technology offers a simple, cost-effective alternative method to produce a volumetric plasma sample that is stable when dried and eliminates requirements for both cold-chain and biohazard transport. The developed analytical workflow appears suitable for advantageous application to pharmacokinetic and toxicokinetic animal studies of edaravone and its metabolites.

1. Introduction

First synthesised in the 1980s as an antioxidant agent for the treatment of brain swelling after a stroke, edaravone (EDR, 5-methyl-2-phenyl-4*H*-pyrazol-3-one, Fig. 1) is still approved for its original indication, but has more recently obtained approval for use against amyotrophic lateral sclerosis (ALS), also known as *Lou Gehrig's disease*. The drug was indeed granted marketing authorisation for ALS in Japan (in 2015 [1]) and then in the USA (in 2017 [2] as an intravenous infusion and in 2022 as an oral solution [3]), under the name Radicava™. It is still one of only three drugs, together with riluzole (1995) [1] and the phenylbutyrate-taurursodiol association (2022) [4], to have reached approval for ALS therapy. In the EU, the marketing authorisation request for EDR was withdrawn by the manufacturer in 2019, following a negative provisional opinion by the European Medicines Agency's (EMA) Committee for Medicinal Products for Human Use (CHMP) [5]; a similar fate befell another prospective anti-ALS agent, masitinib [6].

The mechanism of action of EDR against ALS is still unknown, although oxidative stress is widely considered to be one of the main causes of the neuronal death process typical of ALS. As such, EDR's known free radical scavenging activity [7] could have a neuroprotective effect through oxidative stress reduction. The specific mechanism of EDR radical scavenging is still debated as well [8,9], but it was designed as a phenol-like compound, which generates an aromatic hydroxyl group by keto-enol tautomerism (Fig. 1) [10]. In vivo, at physiological pH values, the dissociated EDR anion can easily transfer an electron to radicals, producing the EDR radical and interrupting chain oxidations (e.g., of lipids). The EDR radical, in turn, can be further oxidised to the

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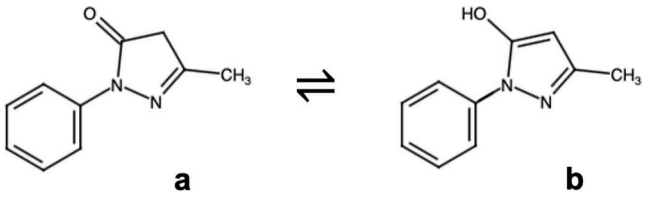

Fig. 1. Chemical structures of the ketone (**a**) and enol (**b**) forms of edaravone.

corresponding peroxide radical and then transformed into EDR 4, 5-dione (Fig. 2) [11].

After administration, EDR undergoes rapid biotransformation ($t_{½}$ = 4–6 h) to the inactive sulphate (EDR *O*-sulphate, EDRS, Fig. 3a) and glucuronide (EDR *O*-β-D-glucuronide, EDRG, Fig. 3b) metabolites. EDRS is the main species found in plasma, while EDRG is predominant in urine [12].

In order to study the behaviour and pharmacokinetics of EDR in rats, a microsampling approach would be very advantageous. Microsampling protocols for animal models use minute amounts of biological fluids (in this case, plasma) and avoid the need for sacrifice and exsanguination, or the severe discomfort for animals due to repeated draws of significant percentages of their circulating blood. This is perfectly in line with the tenets of the 3 Rs principle (Replacement, Reduction and Refinement) principles of animal research [13], greatly reducing the number of animals needed to perform pharmacokinetics studies and also providing more reliable results by reducing inter-subject variability. Moreover, microsampling is necessarily coupled to miniaturised sample preparation procedures, and both are fundamental steps in the implementation of greener analytical methods in line with Green Sample Preparation (GSP) and Green Analytical Chemistry (GAC) guidelines [13,14]. Dried microsampling, where the sample is dried immediately after sampling, provides further advantages, such as increased analyte stability, easier and cheaper storage and shipping conditions, and reduced contamination risks for both samples and personnel.

Although dried blood spot (DBS) on cellulose cards is the most widespread and best-known blood microsampling technique [15–17], in this study volumetric plasma separation cards (vPSC, Telimmune®) were chosen to develop an HPLC-MS/MS analytical protocol for the determination of EDR, EDRG and EDRS levels in rat plasma after EDR administration. Indeed, with this procedure plasma microsampling is carried out by depositing a blood drop on the card and obtaining a volumetrically accurate 3-μL dried plasma spot (vDPS) on a separate card position [18]. This contrasts with DBS, for which sampled blood volume can vary significantly according to haematocrit value, prick size and zone, time and intensity of contact with the card, and other conditions [19,20]. Without proper sampling volume control, analyte quantification in DBS can be problematic, while vDPS has largely overcome this hurdle, also producing plasma spots which provide results immediately comparable to those of common plasma sample analysis, and which can be handled with greater ease than the blood spots provided by classic DBS [21,22].

Just a few published papers include the determination of EDR, EDRG and EDRS in biological fluids, and none of them are predominantly analytical works [23–27]. Therefore, most of them only include scant

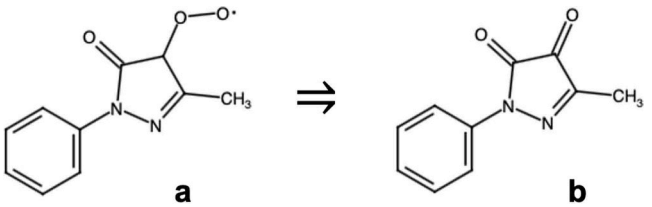

Fig. 2. Sequential edaravone oxidation to the corresponding peroxide (**a**) and 4,5-dione (**b**).

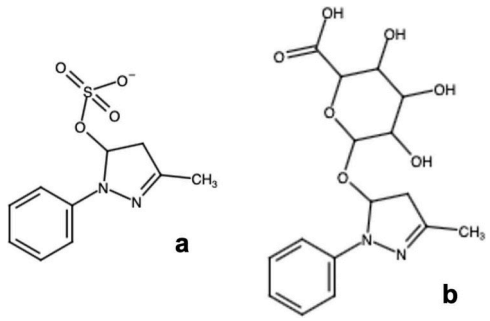

Fig. 3. Chemical structures of the sulphate (**a**) and glucuronide (**b**) edaravone metabolites.

details on the operating conditions and performance of the respective methods. Those that do use two different analytical methods, one for EDR and EDRS, and one for EDRG. Moreover, EDRS is usually determined as EDR after hydrolysis, and this approach requires two different injections of the same original sample (one with and one without hydrolysis), thus leading to significant time, money and reagent waste. To the best of our knowledge, dried microsampling protocols have never been applied before for the analysis of EDR and its two main metabolites. In the following, a single, original HPLC-MS/MS method coupled to vPSC is described for the simultaneous determination of EDR, EDRG and EDRS in rat plasma, where all analytes are quantified as such, without the need for supplemental hydrolysis and re-analysis steps.

2. Experimental

2.1. Chemicals and solutions

EDR, EDRG, EDRS triethylammonium salt and internal standards (ISs) EDR-D_5 (IS1) and EDRG-D_5 sodium salt (IS2) were acquired from Cymit Quimica (Barcelona, Spain). Methanol (MeOH) for LC-MS (≥99.9 %) and formic acid (FA) for LC-MS (98–100 %) were obtained from Merck Italia (Milan, Italy). Ultrapure-grade water (18.2 MΩ cm) was obtained in-house from a Millipore (Milford, MA, USA) MilliQ Gradient apparatus. Stock solutions of individual powder compounds (1 mg/mL) were prepared in MeOH. Working standard solutions in MeOH of all analytes and ISs were prepared daily from stock solutions and stored at −20 °C in amber vials.

2.2. HPLC-MS/MS system

The HPLC-MS/MS system consisted of a Waters (Milford, MA, USA) Alliance e2695 chromatographic pump coupled to a Waters Micromass Quattro Micro triple quadrupole mass spectrometer, through an electrospray ionisation source operating alternately in positive mode (ESI+) for EDR and EDRG and in negative mode (ESI–) for EDRS. Data acquisition was performed in multiple reaction monitoring mode (MRM) with the following optimised parameters: ionisation source voltage, 2.40 kV; ionisation source temperature, 120°C; desolvation temperature, 300°C; desolvation gas flow (nitrogen), 450 L/h; argon was used as collision gas. The dwell time was set to 300 ms for each analyte and IS transition. Precursor and product ions, cone voltage and collision energy values optimised ad hoc for each analyte and IS are reported in Table 1. Data acquisition and processing were performed using Waters MassLynx 4.1 software.

The stationary phase used for chromatographic separations is a Waters Sunfire C18 column (50 ×3.0 mm I.D., 3.5 μm particles), equipped with a C18 guard column (5 ×3.0 mm I.D., 3.5 μm particles). The mobile phase consisted of a mixture of 0.1 % formic acid in MeOH (component A) and 0.1 % formic acid in water (component B) flowing under the following automatic composition gradient program: 0 min,

Table 1
MRM transitions, cone voltage, collision energy and retention time (t_R) for each analyte and IS.

Compound	Q1 (*m/z*)	Q3 (*m/z*)	Cone voltage (V)	Collision energy (eV)	t_R (min)
EDR	175.07	133.1	35	17	5.1
EDRG	351.10	175.1	40	35	2.4
EDRS	253.08	175.1	35	22	3.6
IS1	180.08	138.1	35	17	5.1
IS2	356.12	180.1	40	35	2.4

35 % A; 4.0 min, 85 % A; 7.5 min, 85 % A; 9.6 min, 35 % A; 13.5 min, 35 % A. Complete analyte separation took less than 6 min, while the full chromatographic run lasted 13.5 min, including column reconditioning. Autosampler-injected volume was 10 μL and the flow rate was kept constant at 300 μL/min.

IS1 was used as the IS for both EDR and EDRS, since no deuterated form of EDRS is currently commercially available.

2.3. Microsample collection and pretreatment

The proposed miniaturised sampling strategy was applied to blank rat blood for method development and validation.

For these purposes, blank rat blood was used from residual samples of previous studies. No additional animals were used specifically for this. Sprague-Dawley rats (weighing 180–250 g) were housed in cages under controlled temperature (25 ± 2°C) and relative humidity (45–65 %). Ethical oversight and approval were obtained for all animal-related experiments. For preliminary assays and method validation, blank blood samples (about 200 μL each) were drawn from the caudal vein and collected into heparin-containing tubes.

Aliquots of 90 μL of blank blood were fortified with 5 μL of a standard solution containing the analytes at known concentrations (and ISs at constant concentrations). The obtained fortified blood samples were then subjected to microsampling, pretreatment and HPLC-MS/MS analysis.

vPSC, marketed under the Telimmune® trade name (formerly known as Noviplex), were obtained from Telimmune (North Webster, IN, USA). Sampling is performed by depositing two blood drops onto the card sampling spot, in order to allow uniform absorption. The card is then left to rest for 3 min while the blood drop migrates to the plasma spot formation location (Fig. 4). Afterwards, the card was subjected to forced drying, which was carried out by blowing filtered air, dried by a dehumidifier (30 % RH, 0.10 kg/s flow rate), on the wet microsample for 20 min. The card was then stored at room temperature (RT) in a polyethylene resealable bag containing a desiccant silica gel packet. At the time of analysis, the vDPS is punched out from the card and placed inside a vial. Analyte desorption from the vDPS is performed with 50 μL of 0.2 % FA in MeOH, applying vortex-assisted extraction (VAE) for 1 min and then ultrasound-assisted extraction (UAE) at RT for 8 min. The extract is centrifuged at 3200 rpm for 5 min at 5°C, then the supernatant is quantitatively transferred into a new vial and analysed by HPLC-MS/MS as such.

2.4. Method validation

Validation was carried out according to current international guidelines drafted by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) [28].

2.4.1. Calibration curves

Aliquots of 5 μL of analyte standard solutions at seven different concentrations, containing the ISs at constant concentrations, were added to blank fluid blood prior to sampling. The resulting fortified vDPS samples were subjected to the previously described pretreatment procedure and injected into the HPLC-MS/MS system. The procedure was carried out in triplicate for each concentration. The analyte-to-IS peak area ratios were plotted against the corresponding concentrations of the analytes (expressed as ng/mL) and the calibration curves set up by means of the least-square method. The values of limit of quantitation (LOQ) and limit of detection (LOD) were calculated as the analyte concentrations, which give rise to peaks whose heights are 10x and 3x the baseline noise, respectively.

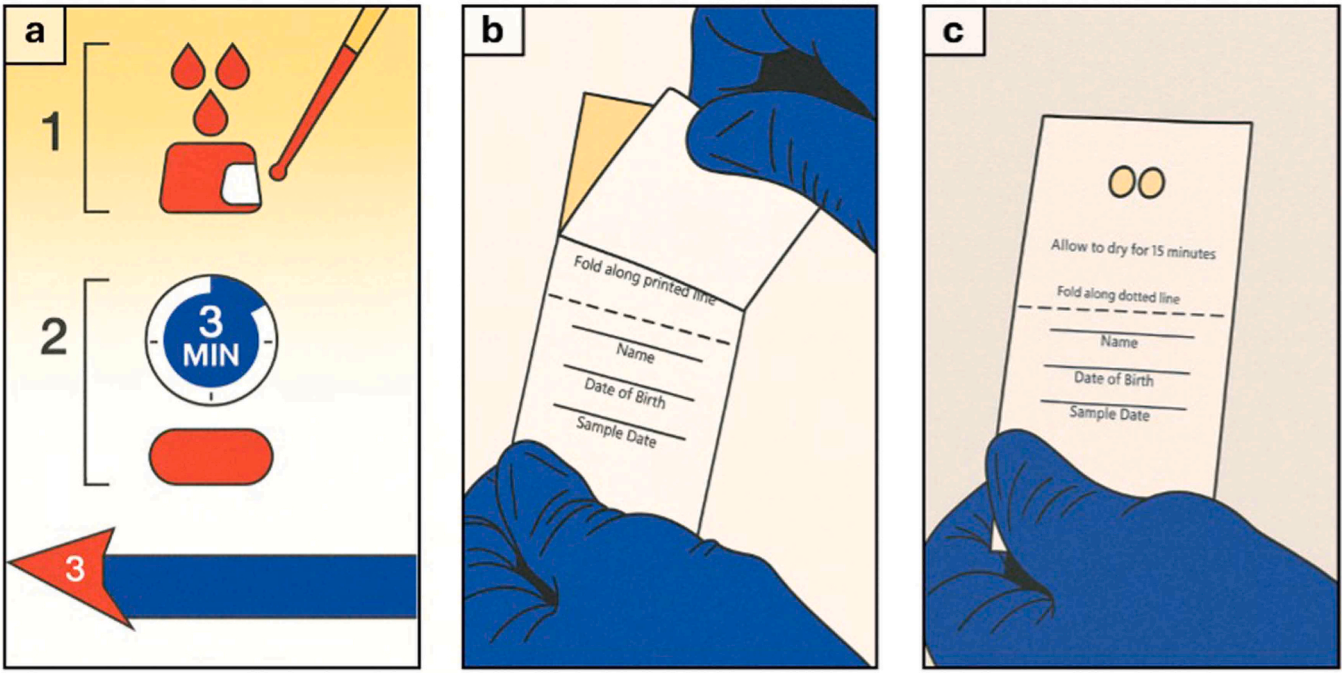

Fig. 4. Plasma microsampling process using vPSC. (a) Two blood drops are applied to the sampling area, initiating plasma separation. (b) After 3 min, the top layer is removed to expose two volumetrically accurate DPS samples (3 μL each). (c) The spots are left to dry before pretreatment and analysis or storage/transportation.

2.4.2. Extraction yield and precision assays

Absolute extraction yields (uncorrected for IS) and precision were evaluated by adding known amounts of the analytes (at three different concentrations, corresponding to the lower limit, a middle point and a high value of each calibration curve) to blank fresh blood, then spotting it on the vPSC and subjecting the resulting vDPS to the sample pretreatment and injecting them into the HPLC-MS/MS system.

The analyte peak areas were compared to those obtained by injecting standard solutions at the same theoretical concentrations and extraction yield was calculated. The assays described above were repeated six times within the same day to obtain intraday precision (repeatability) and on six separate days to obtain interday precision (intermediate precision), both expressed as relative standard deviation (RSD%).

2.4.3. Matrix effect

Matrix effect was assessed by fortifying known amounts of the analytes (at 3 different concentrations, corresponding to the lower limit, a middle point and a high value of each calibration curve) to pretreated blank matrices, immediately before injection. The peak areas obtained from these assays were compared to those of the standard solutions and the corresponding percentage relative error (RE%) values were calculated.

2.4.4. Selectivity

Blank vDPS samples from six different blood batches were subjected to the pretreatment procedure and injected into the HPLC-MS/MS system. The resulting chromatograms were checked for possible interference from endogenous compounds. The acceptance criterion was no interfering peak higher than an analyte or IS peak corresponding to its LOD.

2.4.5. Comparison with conventional plasma samples

Fluid blood was centrifuged, and plasma was separated. Aliquots of 100 μL of plasma were fortified with 5 μL of standard solution containing analytes and/or ISs at known concentrations; the vial was mixed by vortex agitation. The solution was then subjected to microextraction by packed sorbent (MEPS) pretreatment in an SGE Analytical Science (Melbourne, VIC, Australia) C8 barrel insert and needle (BIN) assembly set up in an SGE eVol XR digital analytical syringe apparatus. The BIN was activated with 100 μL of MeOH drawn and discarded 3 times, then conditioned 3 times with 100 μL of water. The fortified plasma sample was loaded in the BIN with 10 draw/discharge cycles at a speed of 5 μL/s; BIN washing was then performed by 3 draw/discharge cycles with 50 μL of 50 mM, pH 7.4 phosphate buffer/MeOH (90/10, V/V) mixture at 20 μL/s and finally the analytes and the ISs were eluted by 5 draw/discharge cycles with 100 μL of MeOH at 5 μL/s. The eluate was brought to dryness under reduced pressure, re-dissolved in 50 μL of solvent A / solvent B (10/90, V/V) mixture and analysed by the proposed original HPLC-MS/MS method. The results obtained from fluid plasma samples were then compared to those obtained from the corresponding vDPS samples.

2.4.6. Stability

To evaluate analyte stability in the dried matrices, blank samples were fortified with the analytes at two concentration levels and then analysed at regular intervals until 60 d from sampling. During this time, vDPS were stored at RT, in a dark and dry place (under both relative humidity and temperature monitoring). The results obtained after IS normalisation were compared to those of fortified samples analysed immediately after sampling and drying.

Comparison with analyte stability in fluid plasma stored at −20°C and pretreated according to the procedure of Section 2.4.5. was also carried out.

2.4.7. Carryover, dilution integrity and robustness

Carryover was evaluated by injecting a 0.2 % FA solution in MeOH immediately after the end of an analytical run of a blank DPS spiked with each analyte at the respective upper limit of quantitation (ULOQ). All chromatographic peaks potentially interfering with any analyte were then quantified and expressed as a percentage of the respective mean LOQ peak area.

Dilution integrity was tested by spiking blank samples at concentrations corresponding to two, five and ten times the ULOQ of each analyte and spotting them on cards. After extraction as described in Section 2.3, the final solution was diluted with the same solvent (0.2 % FA in MeOH) by a factor four, ten and twenty, respectively, and injected. Mean recovery of the theoretical concentrations was calculated.

Robustness was evaluated by making small, deliberate changes to some key HPLC-MS/MS method parameters, namely: FA concentration (0.05–0.15 %); mobile phase component ratio (changing the MeOH ratio by ±5 % at every gradient step) and flow rate (0.25–0.35 mL/min). Extraction parameters were changed as well: extraction volume (40–60 μL), VAE time (0.9–1.1 min) and UAE time (7–9 min).

2.4.8. Accuracy

IS-corrected accuracy was evaluated by means of recovery assays [28]. Known amounts of the analytes at three levels (corresponding to the lower limit, the middle point and a high value of each calibration curve) and of the ISs at constant levels were spotted onto cards already spotted with blood samples from animals receiving EDR at different doses (and dried). The resulting DPS were extracted and analysed. The IS-corrected recovery (%) of each fortified analyte was calculated. These assays were repeated three times during the same day to calculate the corresponding SD data.

3. Results and discussion

3.1. Preliminary assays

3.1.1. Chromatographic assays

Several chromatographic parameters, such as sorbent material, FA concentration, solvent ratio gradient, and flow rate, were tested to obtain analyte separation within acceptable run times and to maximise MS ionisation efficiency. Endcapped and non-endcapped C8, C18 and phenyl sorbents were tested; gradient percentage of solution B was changed in the 20–90 % range and gradient times were changed from 3 to 10 min; flow rate was tested between 0.05 and 0.3 mL/min. Ammonium formate and FA as mobile phase additives were tested in the 0.02–0.50 % range.

Under the optimised conditions reported in Section 2.2., all analytes were successfully eluted and separated in less than 8 min, yielding efficient and symmetrical peaks.

3.1.2. Evaluation of sampled volume

Gravimetric tests were performed to define the accuracy of the volume of whole blood sampled by the vPSC technology. Card plasma spots (n = 6) were punched out and weighed using a balance with a resolution of 0.01 mg, before and after normal sampling and the results were compared with the weight of 3 μL of rat plasma, drawn with a micropipette and spotted on the punched-out card. Plasma collected by depositing blood on vPSC gave results that were not statistically different from plasma pipetted on the cards (3.0 ± 0.1 μL), thus demonstrating the accuracy of the Telimmune device.

3.1.3. Evaluation of forced drying time

Common procedures for the drying of blood or plasma microsamples require about 60 min at RT. As demonstrated in previous studies [29,30], forced drying of biological fluids by reduced RH air blowing can significantly shorten these times.

Preliminary drying time assays by gravimetric analysis (n = 3) were carried out by measuring every 5 min the changes in weight of the punched-out plasma spot subjected to a constant air stream (30 % RH,

0.10 kg/s flow rate, RT), using a balance with a resolution of 0.01 mg, until at least three consecutive readings were constant. This showed that the time required to produce a constant weight vDPS under these conditions is 20 min (Fig. 5). Thus, using the forced-air drying procedure allows the operator to spare about two-thirds of the usual drying time.

3.1.4. Sample preparation procedure development

Several different extraction solvents and mixtures thereof were tested for the extraction of analytes and ISs. Pure MeOH, ACN, water and their mixtures in different ratios (from 3/1–1/2, V/V) were tested, as well as the same solvents and mixtures acidified with FA at different concentrations (0.05–0.5 %). Best preliminary results were obtained applying acidified pure organic solvents, i.e., with 0.2 % FA in MeOH and 0.2 % FA in ACN, with slightly better data for MeOH (extraction yields higher than 82 % and 79 %, respectively). Three different extraction assistance methods were also tested, i.e., UAE (1–10 min), VAE (1–5 min) and microwave-assisted extraction (MAE, 10–100 s). Good extraction efficiency was obtained using either UAE or MAE, and the results were made even better by combining either technique with VAE. Extraction with FA in MeOH using VAE+UAE provided the best results overall.

Chromatograms of a blank vDPS sample fortified with the analytes and the ISs, after sample pretreatment and HPLC-MS/MS analysis, is shown in Fig. 6. As one can see, satisfactory resolution, peak symmetry and run times were obtained, coupled to stable baseline and viable baseline noise.

3.2. Method validation

3.2.1. Linearity and sensitivity

Calibration curves produced satisfactory results for all analytes within the following linearity ranges: 2–2000 ng/mL for EDR, 5–5000 ng/mL for EDRS and EDRG. Calculated correlation coefficients (r^2) were always higher than 0.9990.

LOD and LOQ values were calculated for each analyte; LOQ ranged from 2.0 to 5.0 ng/mL and LOD ranged from 0.7 to 1.7 ng/mL (Table 2).

3.2.2. Extraction yields and precision

Extraction yield assay results were good at all three concentration levels and for all analytes, always within the 82–93% range.

Results were also good for both intraday and interday precision, being RSD always lower than 14.1 % (again for all concentration levels, including the LOQ level, and all analytes). Complete results are reported in Table 2.

3.2.3. Matrix effect

Matrix effect was very low: mean percentage relative error (RE%) was always lower than 7.5 % for EDR, 7.8 % for EDRS and 7.2 % for EDRG (as one can surmise from Table 2). This testifies to the good purification level achieved despite the simple and straightforward analyte extraction procedure.

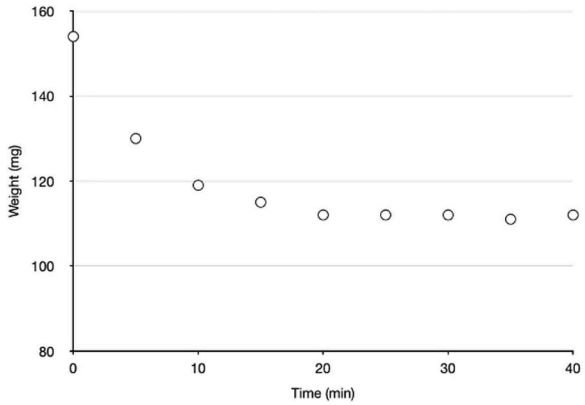

Fig. 5. Drying time assay results for blank vDPS.

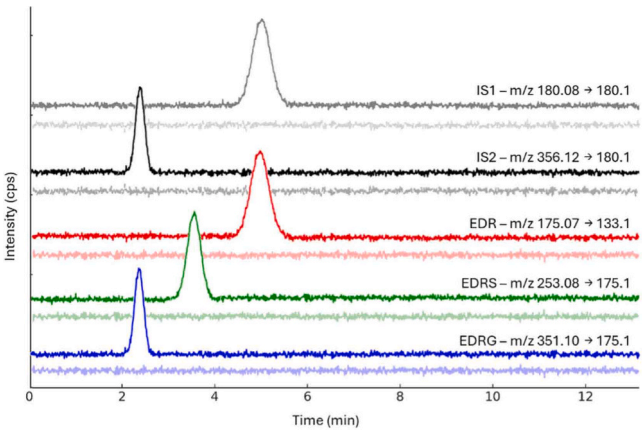

Fig. 6. HPLC-MS/MS MRM chromatogram from a vDPS sample: fortified with analytes and ISs (200 ng/mL) vs blank.

3.2.4. Comparison with conventional plasma samples

To confirm the reliability of the vDPS workflow, the same fortified blank blood samples were used to produce both vDPS and fluid plasma samples. Both kinds of samples were then pretreated and analysed as described in the respective sections of the Experimental.

Excellent agreement in terms of accuracy between the two matrices was always obtained, since the results obtained from vDPS were not statistically different from those obtained from plasma (Table 3).

3.2.5. Stability

Generally speaking, analytes are usually much more stable in dried microsamples than in the biological fluids from which they are prepared, thanks to the absence of water, which stops most chemical and enzymatic reactions [31]. This often allows laboratories to store DPS at RT for months without appreciable analyte degradation. In this study, stability was assessed over 60 days, testing stored blank fortified vDPS every 6 days. During this period, relative humidity in the storage containers varied between 20 % and 35 %, while temperature ranged from 22 to 29°C. Results were compared to those of blank fortified plasma stored at −20°C. The results reported in Table 4 show analyte stability in vDPS is really outstanding, even though they were not subjected to low-temperature storage. Stability in vDPS at RT was comparable to that in plasma at controlled freezing temperature but with obvious advantages in terms of practicality, costs, space and analysis reliability. In any case, analyte recovery from vDPS at the endpoint was always higher than 91 %. This also ensures that no “dried plasma ageing” phenomena occur over the tested timeframe, where analyte extraction becomes progressively more difficult due to their increasing interaction with the cellulose support.

3.2.6. Carryover, dilution integrity and robustness

Detailed results for these validation parameters are reported in Supplementary Tables S1-S2.

Briefly, carryover was almost negligible for all analytes, with maximum interfering peaks always lower than 15 % of the respective LOQ area. Analyte recovery values obtained from diluted sample extracts were substantially identical to those obtained from undiluted extracts. Every operating condition change made during robustness assays produced results (in terms of analyte quantitation) that were always within ±10 % from those obtained under optimal experimental conditions.

Table 2
Validation results on blank fortified samples.

Compound	Linearity range (ng/mL)	r^2	LOQ (ng/mL)	LOD (ng/mL)	Level (ng/mL)	Extraction yield (%)[a]	Precision (RSD%)[a]		Matrix effect (RE% ± SD)[a]
							Intraday	Interday	
EDR	2–2000	0.9994	2.0	0.7	2	89	12.2	14.0	7.4 ± 0.1
					500	90	4.4	5.3	3.8 ± 0.2
					2000	93	0.6	0.9	0.3 ± 0.1
EDRS	5–5000	0.9991	5.0	1.7	5	88	10.9	11.7	7.7 ± 0.3
					2000	89	0.5	0.8	1.4 ± 0.2
					5000	90	0.3	0.5	0.6 ± 0.1
EDRG	5–5000	0.9994	5.0	1.7	5	85	9.8	10.2	7.1 ± 0.4
					2000	84	0.6	1.0	1.6 ± 0.2
					5000	82	0.1	0.3	0.8 ± 0.1
IS1	/	/	/	/	30	93	3.0	3.3	2.1 ± 0.1
IS2	/	/	/	/	30	90	3.4	3.8	1.4 ± 0.2

[a] n = 6.

Table 3
Result comparison between fortified blank vDPS and fluid plasma samples.

Concentration level	Mean concentration found (ng/mL)[a]					
	EDR		EDRS		EDRG	
	vDPS	Plasma	vDPS	Plasma	vDPS	Plasma
Low	2.2 ± 0.2	1.9 ± 0.1	5.0 ± 0.3	5.5 ± 0.1	4.8 ± 0.2	5.1 ± 0.2
Middle	504 ± 9	490 ± 5	1983 ± 11	2001 ± 15	2005 ± 17	2011 ± 8
High	2035 ± 12	2020 ± 15	5054 ± 26	5003 ± 22	5078 ± 20	5099 ± 33

[a] n = 3

Table 4
Stability test results.

Time point (d)	Mean analyte recovery (%)	
	vDPS @ RT	Plasma @ −20°C
0	100	100
6	99	98
12	99	97
18	97	97
24	95	96
30	95	96
36	94	95
42	93	93
48	93	92
54	93	92
60	92	92

3.3. Analysis of real samples and accuracy

Blood samples from 6 different rats treated with EDR were used to produce both fluid plasma and vDPS and both kinds of samples were analysed. Complete results are shown in Table 5 and an example chromatogram obtained from a real vDPS sample is displayed in Fig. 7.

As with blank spiked samples, also for treated rat samples qualitative and quantitative results obtained from vDPS were always in very good agreement with those obtained from fluid plasma.

IS-corrected accuracy was also satisfactory, with mean recovery values of 98 % for EDR and EDRS and 101 % for EDRG.

Table 5
Method application to real vDPS samples and comparison with fluid plasma.

Subject	Concentration found ± SD (ng/mL)[a]					
	EDR		EDRS		EDRG	
	vDPS	Plasma	vDPS	Plasma	vDPS	Plasma
1	153 ± 5	160 ± 3	333 ± 6	340 ± 9	202 ± 2	197 ± 7
2	100 ± 4	94 ± 6	473 ± 9	459 ± 9	421 ± 7	418 ± 7
3	77 ± 5	77 ± 3	201 ± 4	210 ± 8	177 ± 7	182 ± 8
4	228 ± 5	235 ± 8	778 ± 8	790 ± 8	278 ± 3	288 ± 8
5	48 ± 3	47 ± 4	189 ± 2	190 ± 7	104 ± 8	104 ± 4
6	51 ± 3	53 ± 2	451 ± 7	460 ± 5	190 ± 10	192 ± 8

[a] n = 3

4. Conclusion

For the first time, this study has described a promising approach for the analysis of EDR and its two main conjugated metabolites in volumetric plasma microsamples by leveraging a vPSC technology, which uses a combination of paper and calibrated filters to generate volumetrically accurate 3-μL DPS from a few whole blood drops. This innovative workflow facilitates the application of the "3 R" principles in animal studies, allowing scientists to carry out multiple blood sample drawings from the same animal without causing unnecessary suffering, stress and sacrifices, thereby reducing the number of animals needed for studies, including pharmacokinetic and toxicokinetic ones. Moreover, the dried microsampling approach needs much lower amounts of materials and expensive reagents and solvents than the corresponding conventional sampling techniques and procedures; it also does not require any controlled temperature for storage and transportation, thus contributing to compliance with current principles of GAC and GSP. Results obtained from vDPS are immediately comparable to those obtained from conventional plasma samples, thus allowing interoperability with most currently available databases and analytical services, whereas common blood microsampling techniques produce results

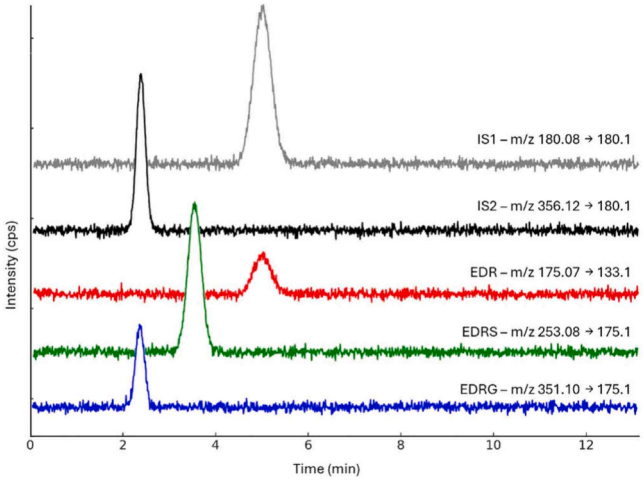

Fig. 7. HPLC-MS/MS MRM chromatogram from a real vDPS sample. EDR, EDRS, and EDRG were quantified at 50, 188 and 98 ng/mL, respectively.

referring to whole blood concentrations, which require conversion to plasma concentration, introducing another source of experimental error and uncertainty due to haematocrit variability. It should be noted that the composition of plasma obtained by filtration (as the one produced by Telimmune cards) can differ slightly from that of plasma obtained by centrifugation (as most conventional plasma samples). However, these differences are mostly considered secondary and, in many cases, do not lead to any discrepancies in the final analytical results, as was the case in this study.

In conclusion, it can be claimed that use of the new Telimmune technique for plasma generation and microsampling coupled to HPLC-MS/MS analysis provides a reliable analytical platform for the determination of EDR, EDRS and EDRG concentrations in rats treated with the drug.

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CRediT authorship contribution statement

Michele Protti: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Roberta Di Lecce:** Validation, Methodology, Investigation, Formal analysis, Data curation. **Jiri Adamec:** Writing – review & editing, Resources. **Regazzoni Luca Giovanni:** Resources, Investigation. **Valeria Valsecchi:** Resources, Investigation. **Claudia Volpi:** Resources, Project administration, Investigation, Funding acquisition. **Roberto Mandrioli:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Data curation, Conceptualization. **Laura Mercolini:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jiri Adamec is co-founder of Telimmune but was not involved in any aspect of the experimental design and data interpretation.

Roberto Mandrioli, given his role as Advisory Editorial Board member in J. Pharmaceutical and Biomedical Analysis Open, had no involvement in the peer review of this article and had no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to another journal editor.

All other authors declare no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpbao.2025.100094.

Data availability

Data will be made available on request.

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