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Quantitative analysis of methyl and propyl parabens in neonatal DBS using LC–MS/MS

Aim: Excipients are used to overcome the chemical, physical and microbiological challenges posed by developing formulated medicines. Both methyl and propyl paraben are commonly used in pediatric liquid formulations. There is no data on systemic exposure to parabens in neonates. The European Study of Neonatal Exposure to Excipients project has investigated this. Results & methodology: DBS sampling was used to collect opportunistic blood samples. Parabens were extracted from the DBS and analyzed using a validated LC–MS/MS assay. Discussion & conclusion: The above assay was applied to analyze neonatal DBS samples. The blood concentrations of parabens in neonates confirm systemic exposure to parabens following administration of routine medicines.

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Keywords: acceptable daily intake • DBS • excipient kinetics • internal standard • parabens • solid phase extraction

Pedicratic liquid formulations contain the active pharmaceutical ingredient and excipients. To overcome the physical, chemical and microbiological challenges in these formulations, excipients are normally used. The commonly used excipients in pediatric formulations are methyl (MPB) and propyl paraben (PPB), which are the methyl and propyl esters of p-hydroxybenzoic acid, respectively Figure 1. Acceptable daily intake of MPB has been established for adults at 10 mg/kg bw/day [1]. No acceptable daily intake has been assigned to PPB. Until recently, there has been no data on systemic exposure to parabens in human neonates. There are long standing concerns about parabens relating to the possibility of oestrogenic effects and cell physiology, which could cause adverse long-term outcomes. Previous work assessments of exposure to excipients in neonates have been based on estimates of intake [2]. In order to move from theoretical extrapolations of exposure based on estimates of intake, the European Study of Neonatal Exposure to Excipients (ESNEE) project conducted studies within a clinical environment to define systemic exposure of MPB and PPB in neonates. An observational study of systemic concentrations of parabens in human neonates could provide a good indication as to the range of exposures in neonates during their routine clinical care.

To study the PK of drugs, the biological matrix of choice is blood plasma. Quantification of drugs or excipients in the blood samples taken from neonates is very challenging. The total circulating volume of blood [3] in preterm and term neonates is very low (80–90 ml/kg or ∼45–360 ml). The DBS sampling approach was used in this study for clinical and ethical reasons, as multiple plasma sampling (which is a prerequisite for PK studies) is not always possible in this age range. Emmons and Rowland have discussed the application of DBS to study PK [4].

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Parabens have been quantified in a range of matrices using high performance liquid chromatography coupled with the following detectors; UV [5–8], photodiode array [9] and MS/MS [10–22]. GC has also been used with free induction decay [23], TOF [24] and MS [25] as has thin layer chromatography [10]. The biological matrices used included urine [5, 12, 14, 16, 18–19, 22–24], serum [13, 19, 22, 24], seminal plasma [14], breast tissue [10], placental tissue [15], saliva [6], breast milk [13, 21] and rat plasma [20]. Parabens have also been determined in water [24, 25], personal care products [6, 8, 11, 12, 26] and pharmaceutical preparations [7, 9].

Several sample preparation methodologies have been reported to facilitate the extraction of parabens from the above tissues. These approaches include SPE [12–13, 19, 22, 24], LLE [8, 14, 16, 18, 20, 21, 23], dispersive LLE [17], solvent extraction [5–6, 10, 15, 26], stir-bar sorptive extraction [25], super critical extraction [11] and simple protein precipitation [20].

The first person to use the DBS technology in infants to screen in-born errors of metabolism was R Guthrie [27]. In comparison to the venous sampling, the DBS technique has proven to be minimally invasive. This sampling technique can be used at home or in the clinical settings, as it requires less training. Storing and shipping these samples in dried form is very safe [28, 29]. Our group has previously reported similar applications involving DBS [30–34] to develop bioanalytical methods.

Herein, we report the development and validation of an assay to support the clinical measurement of MPB and PPB from DBS by LC–MS/MS. This is the first report describing the quantification of parabens in the DBS samples.

Materials & methods

Chemicals & reagents

Analytical grade reagents and chemicals were used for all the extractions and assays. Methyl, propyl and benzyl paraben (BPB) (Figure 1) were purchased from Sigma (Poole, UK). BPB was used as an internal standard (IS). HPLC grade solvents and water, buffers, Oasis™ HLB cartridges (1 cc/30 mg) were all purchased as mentioned in our previous publica-

Figure 1. Chemical structures of parabens used in the study.

tion [32]. The School of Pharmacy Ethical Committee (013PMY2009) approved the collection of blank blood from healthy human volunteers.

Ahlstrom 226 (Guthrie cards) together with storage pouches were purchased from 3M Security Systems Division (Oldham, England). A 8 mm single hole punch (2700–62) used to cut out the DBS disks from the cards was purchased from Darice, Strongsville, Ohio, USA. Zip-loc bags, silica gel pouches and freezer storage lunch boxes were procured from the Amazon website. Manual SPE manifold (Waters Dublin, Ireland) was used for SPE. For solvent evaporation, Zymark Turbo Vap® LV Evaporator workstation (Zymark, Runcorn, UK) was used. Eppendorf tubes 2 ml (Sarstedt, Numbrecht, Germany) were used for the initial extraction of the DBS samples. Stuart rotator SB2 and vortex mixer SA8 (Bibby Scientific, Staffordshire, UK) were used to mix the spiked blood and vortex samples, respectively.

Chromatography

Waters Alliance HT system 2795 (Waters, Dublin) was the LC system used for separation. A XBridge™ C18 column (3.5 μm, 4.6×100 mm) was used along with XBridge (3.5 μm, 4.6 × 20 mm) guard column, which had a matching chemistry. Both the columns were maintained at 25°C during the chromatographic separation. Mobile phase consisted of methanol/5 mM ammonium acetate (99:1 v/v) with a flow rate of 0.3 ml/min.

Mass spectrometry

The above LC system was hyphenated to Waters Micromass Quattro Premier™ tandem quadrupole (Waters, Manchester, UK).

The MS conditions for the electrospray negative ion mode (ESI-) were optimized at the following conditions: Cone gas (nitrogen) 75 l/h, desolvation gas (nitrogen) 800 l/h, desolvation temperature 400°C, source temperature 145°C and capillary voltage 2.60 V. Argon gas was used as the collision gas.

The following multiple reaction monitoring transitions were setup for quantitation of each ion:

- **MPB**: For molecular ion, quadrupole 1 was set at m/z 150.90 and for product ion quadrupole 2 at m/z 91.80. The cone voltage and collision energy for the product ion, the detector was set at 20 V and 25 eV, respectively.

- **PPB**: For molecular ion, quadrupole 1 was set at m/z 178.96 and for product ion quadrupole 2 at m/z 91.80. The cone voltage and collision energy for the product ion, the detector was set at 23 V and 26 eV, respectively.
• BPB (IS): For molecular ion, quadrupole 1 was set at m/z 227.00 and for product ion quadrupole 2 at m/z 91.80. The cone voltage and collision energy for the product ion, the detector was set at 25 V and 24 eV, respectively.

For each ion the dwell time was set at 0.05 s.

Software
MassLynx™ 4.1 Software was used to control the LC system and the mass spectrometer and the data were processed using QuanLynx Application Manager.

Sample preparation
Preparation of spiked standards & working solutions
The stock (1 mg/ml) and working standards for MPB, PPB and BPB were prepared in methanol. The working calibration standards of MPB and PPB (prepared and mixed in the same pot) were prepared at the concentrations 50, 25, 20, 5, 4, 2.5 and 1 μg/ml. Similarly MPB and PPB (prepared and mixed in the same pot) working quality control (QC) standards were prepared separately at concentrations 40, 10, 2 and 1 μg/ml. A 5 ng/ml working standard of IS was prepared. All the above solutions were stored at 4°C and were brought to (20°C) room temperature, before use.

Spiking of whole blank blood for the preparation of calibration & QC samples
Calibration standards: to 980 μl of whole blood, 20 μl of the appropriate MPB + PPB working standard solution containing 50, 25, 20, 5, 4, 2.5 and 1 μg/ml was spiked to give final concentrations of 1000, 500, 400, 100, 80, 50 and 20 ng/ml.

Similarly, the QC samples at concentration 20 ng/ml (LOQ), 40 ng/ml (low QC), 200 ng/ml (middle QC) and 800 ng/ml (high QC) were prepared by spiking 20 μl of MPB working solutions 1, 2, 10 and 40 μg/ml to 980 μl of whole blood, respectively.

Blood spotting
Spiked blood was mixed very slowly by rotating for 45 min at room temperature before spotting. This was done for the equilibration of the parabens in the blood. The blood spots were prepared by accurately transferring 15 μl of the spiked blood, using a calibrated pipette from both (calibration and QC standards) onto Ahlstrom 226 cards. The samples were dried for 3 h at room temperature in the dark (cupboard) and then placed in zip lock bags along with silica gel pouches. The cards were then stored in polypropylene freezer storage sealed containers at -20°C until analysis.

Extraction procedure for DBS samples
The DBS samples were brought to room temperature before analysis. An 8 mm disk was punched from the card, wherein the complete blood spot was captured. This was then transferred to 2 ml eppendorf tubes and extracted by addition of 1 ml of methanolic BPB (IS) solution (5 ng/ml), followed by vortex mixing for 30 min. The extracts were then evaporated to dryness under nitrogen on the Zymark Turbovap at 37°C and reconstituted with 1 ml of milli-Q water, followed by vortex mixing for 1 min. The samples were further cleaned using SPE Oasis® HLB cartridges. The clean up was done as mentioned in our previous publication [32] and reconstituted in 200 μl of methanol. A volume of 20 μl was injected with the auto sampler temperature set at 15°C.

ESNEE clinical study & sample collection
The Ethical approval for the ESNEE study was granted by National Research Ethics Service Committee North West - Greater Manchester North, ref no 11/NW/0665. The samples in Estonia were collected under the ethical approval from the University of Tartu (210/T-11). DBS sampling was used to collect opportunistic blood samples (15 μl) from the neonates who had been administered parabens containing formulations. A total number of 927 DBS samples were collected from 196 neonates from 4 UK and 1 site from Estonia. The study was registered with International Standard Randomised Controlled Trial (ISRCTN 31837223).

Method validation
US FDA guidelines [35] were applied for all the validation experiments.

Six different sources of the blank DBS samples were used to determine the selectivity. To assess linearity, five calibration curves were analyzed on five consecutive days. The slopes, intercepts, correlation (r), the weighting factor (1/X²) fitted for the calibration curves by least-squares linear regression and the LOQ were all calculated as previously reported [32]. Accuracy (%RE) and precision (%RSD) were calculated by analyzing five replicate sets on five separate occasion, of each of the four concentrations (20, 40, 200 and 800 ng/ml) of the QC samples.

The matrix effect (ME) of parabens and IS was monitored at three different concentrations (20, 200, 800 ng/ml) and was calculated by comparing the responses from postextracted spiked samples to the pure solutions. The ratio <85% or >115% implies an exogenous ME, if the ratio equals to 100, it implies the absence of ME. Whereas, ME (%) below or above 100 indicates ion suppression or enhancement, respectively.
The MS response (pre-extracted to postextracted spiked sample) into a blank matrix was compared with, to determine recovery. Stability of MPB, PPB and IS in stock solution was tested at the storage temperature of 4°C (for 1, 3 and 6 months) and stability of the stock solutions was tested at room temperature for up to 24 h at 20°C. This was done by comparing the area response with those of freshly prepared stock solution. Stability of the spiked DBS samples stored at -20°C was assessed over a period of 24 h, 1, 3, 6 months and 1 year. For this, freshly spiked DBS samples were extracted and the ratio of peak areas (parabens to IS) was compared with the stored DBS samples. Since this was the first time any study was carried out to analyze these parabens in the DBS, it was collectively decided by the clinicians and the analysts that the samples (both spiked and patient) will be stored at -20°C on the hospital premises as well as on the analytical laboratory site, until analysis. These samples were very precious, as the number of spots available for the analysis from the neonates were limited.

**Blood plasma partitioning of parabens**

The blood plasma partitioning for both the parabens was performed as per the protocol described by Yu et al. [36]. Two concentrations were chosen 20 and 500 ng/ml for the experiment. Both, spiked reference plasma and the whole blood plasma were incubated at 37°C for 1 h and sampling was done at 0, 10, 30 and 60 min. These samples were then analyzed. The blood plasma ratio was calculated using the equation below. Hematocrit of the blood was measured as soon as it was collected from healthy volunteers.

\[ K_{RBP-PL} = \frac{1}{H} \times \frac{I_{REF-PL}}{I_{PL}} + 1 \]

where \( I_{REF-PL} \) is instrument response for Reference plasma and \( I_{PL} \) is instrument response for whole blood plasma (equilibrating plasma) and \( H \) is the hematocrit.

**Results & discussion**

**Extraction**

Extraction of parabens from the DBS samples was optimized using different solvents or solvent mixtures such as 100% methanol, 100% water, methanol/water (25, 50 and 75%), 100% acetonitrile and acetonitrile/water (25, 50 and 75%). Spiked DBS samples (20, 100 and 800 ng/ml) were extracted in each of the solvents/solvent mixtures for a period of 15, 30, 45, 60, 75, 90 and 105 min. Extracted samples at each time point were analyzed. Results showed that methanol was the best solvent and that 30 min was sufficient to extract the parabens present in the samples.

PPB is structurally and chemically similar to MPB and PPB, hence was used as an IS. No stable-isotope labeled IS was used. SPE was used as a sample clean-up step, to reduce the ME and any interference arising from the endogenous species present in the matrix. The SPE clean-up also showed a two-fold increase in sensitivity of MPB and PPB.

**Metabolites, chromatography & selectivity**

The chromatography showed no interference or ME in both (spiked and patient samples) at the retention times of IS and parabens. The main metabolite of the parabens is p-hydroxy benzoic acid (m/z 138.12). No interference from this metabolite was seen at the multiple reaction monitorings of MPB, PPB and BPB (IS), as the molecular ions were set for m/z 150.90, 178.96 and 227.00, respectively, for quadrupole 1. Some patient samples showed a peak Figure 2, which eluted approximately 1 min before both the parabens. Some drug metabolites or ISs can undergo fragmentation in the source of the mass spectrometer. These substances should be well-separated chromatographically from the parent drug before they reach the ion source for proper quantification. The peak seen in Figure 2 was well separated and was identified as a glucuronide metabolite of the two parabens. Glucuronidation is a common biotransformation mechanism in drug metabolism. It is a well-known phenomenon, that glucuronides undergo in-source dissociation in both positive and negative electrospray ionization. This is mainly induced by cone voltage, due to which the resulting product ions have the same m/z values of precursor ions of the parent analyte [37,38]. Chromatographically if the glucuronide and its parent are not separated, this would interfere with the quantitation, but in our case both of them were well separated and did not hinder the quantitation.

The aim of this observational study was to look at the circulating concentrations of the parent parabens (MPB and PPB) in the neonatal blood samples and hence no attempt was made to quantify these metabolites.

**Linearity, limit of quantification, imprecision & recovery**

The LOQ for both the parabens was calculated at 20 ng/ml. Calibration plots of both the parabens in DBS samples were constructed using weighted (1/X²) linear regression. The% deviation for each calibration points was less than 10%. Quanlynx software was used to perform the quantification of the parabens. The calibration curves for both the parabens were linear over the range 0–1000 ng/ml and a mean r² = 0.995 ± 0.003 (n = 5). For MPB the mean slope was 0.001
and intercept was 0.002, similarly for PPB it was 0.014 and 0.003, respectively.

There was no carryover seen during the analysis, the calibration curve and the QC’s passed the FDA validation criteria [35]. The number of spots available per neonate were 1 or 2 (3, 4 in rare cases), hence the incurred sample reanalysis could not be performed. Based on our pilot study, the clinical data and dosage information, we had an estimation of range of concentrations in the samples, which fell within the calibration curve, hence the dilution test was not performed.

The validation data (inter- and intra-day) for MPB are shown in Table 1 and PPB in Table 2. The data obtained were within the FDA guidelines [35]. Inter- and intra-day variations for both the parabens were established by the analysis of five replicate sets of each of the four concentrations (20, 40, 200 and 800 ng/ml) of the QC samples on five separate occasions. The interday imprecision was between 1.1 and 3.4% for MPB and 1.1 and 4.3% for PPB. The intraday imprecision was between 0.05 and 0.1% for MPB and 0.02 and 0.14% for PPB. The accuracy for both the parabens was well within ±15%. The recovery of both the parabens and IS from DBS was approximately 75%.

Matrix effect

The reproducibility and accuracy of the assay may get affected due to coeluting, endogenous compounds from the matrix, which will indirectly affect the outcome of the kinetic data, hence the FDA have hence indicated in their guidelines [35] to assess ME. The estimated matrix effect (% ME) on both the parabens and IS was found to be within 85% and 115% [39]. Table 3 shows% ME on the parabens and the IS, and clearly indicates that there is no major ion suppression or enhancement on these analytes. The SPE sample clean-up method did help to minimize this effect.
Stability
Stock solution
The stock solution of parabens was stable for 6 months in methanol at 4°C and for 24 h at room temperature.

DBS samples
Spiked DBS stored at −20°C for the period of 24 h, 1, 3, 6 months and 1 year showed values comparable with freshly prepared samples.

Blood plasma partitioning of parabens
For both methyl and propyl parabens, the blood to plasma partitioning ratio (KRBC/PL) was calculated to be 1.0 based on the formula mentioned in methods section. Therefore, both the parabens distributed equally in RBC and plasma. Hence, both whole blood (in our case DBS samples) and plasma (not applicable in our study) could have been used for sampling.

Sample collection from the patients, hematocrit & application of the analytical method to the ESNEE clinical study
The blood taken from the neonates was mostly from heel pricks which was accurately measured using a fixed volume 15 ml capillary device and transferred to the cards. Only two to three spots were available (in some instances only one spot was taken). To deal with the issue of the hematocrit, an 8 mm disk was punched from the card, wherein the whole spot was captured for analysis. This was based on Fan et al.’s findings [40] which discuss how to deal with the hematocrit issue especially with the variation expected in neonatal patient groups. Whole spot methods eliminate the variation from spreading and nonhomogeneity and allow for more consistent DBS concentrations, even at different hematocrit levels as long as the volume of the spotting is accurate. Zheng et al. [41] have also demonstrated that the whole spot approach was effective in eliminating the hematocrit effect for the analysis of apixaban in human DBS when an accurate blood sample volume was collected on DBS cards.

The validated method was applied to the analysis of the above clinical samples. A total of 927 DBS samples from 196 recruited patients (neonates) were analyzed. Parent(s) or guardian(s) of all eligible patients gave informed verbal and written consent. Opportunistic sampling was used to collect samples from each patient, wherein a tiny amount of sample was taken while the bloods were done for routine laboratory analysis. Figure 2 represents a chromatogram of a preterm neonate, 21 days old, 1.5 kg in weight at a sampling time of 16 h postdose of a pediatric formulation containing parabens. The concentrations obtained from the patient sample analysis from each hospital site are shown in the Figure 3. The hospitals who participated in this study were Chester, Leighton (LTGN), LWH (Liverpool Women’s Hospital) and Arrowe Park from UK and University of Tartu hospital from Estonia. Only 40% of MPB and 14% PPB samples yielded the concentrations above the LOQ (20 ng/ml). This primarily reflects the low amounts of MPB and PPB excipients present in pharmaceutical formulations, typically 0.1–0.2% w/v and 0.02% w/v, respectively. It could also reflect rapid metabolism of the parabens [42] in both the gastrointestinal tract and liver.

Conclusion
The aim of the ESNEE was to develop excipient kinetic EK models for selected excipients in neonates. Relatively very few of the medicines administered to neonates are tested in this age group. There is little previous work on the exposure and safety of the excipients within the neonatal population. Hence, to study the

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### Table 1. Precision and accuracy data for methyl paraben.

<table>
<thead>
<tr>
<th>Concentration (ng/ml)</th>
<th>Precision</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>% RSD (CV)</td>
</tr>
<tr>
<td><strong>Interday</strong></td>
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<td></td>
</tr>
<tr>
<td>20 (LOQ)</td>
<td>19.4 ± 0.5</td>
<td>2.5</td>
</tr>
<tr>
<td>40 (LQC)</td>
<td>40.1 ± 1.1</td>
<td>2.7</td>
</tr>
<tr>
<td>200 (MQC)</td>
<td>196.6 ± 6.6</td>
<td>3.4</td>
</tr>
<tr>
<td>800 (UQC)</td>
<td>790.7 ± 8.7</td>
<td>1.1</td>
</tr>
<tr>
<td><strong>Intraday</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 (LOQ)</td>
<td>18.3 ± 1.5</td>
<td>0.08</td>
</tr>
<tr>
<td>40 (LQC)</td>
<td>42.4 ± 4.2</td>
<td>0.1</td>
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<tr>
<td>200 (MQC)</td>
<td>206.0 ± 17.8</td>
<td>0.09</td>
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<tr>
<td>800 (UQC)</td>
<td>768.6 ± 15.8</td>
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### Table 2. Precision and accuracy data for propyl paraben.

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<th>Concentration (ng/ml)</th>
<th>Precision</th>
<th>Accuracy</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>% RSD (CV)</td>
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<tr>
<td><strong>Interday</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 (LOQ)</td>
<td>19.7 ± 0.9</td>
<td>4.3</td>
</tr>
<tr>
<td>40 (LQC)</td>
<td>39.6 ± 0.4</td>
<td>1.1</td>
</tr>
<tr>
<td>200 (MQC)</td>
<td>196.0 ± 4.9</td>
<td>2.5</td>
</tr>
<tr>
<td>800 (UQC)</td>
<td>823.0 ± 18.9</td>
<td>2.3</td>
</tr>
<tr>
<td><strong>Intraday</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 (LOQ)</td>
<td>18.0 ± 2.7</td>
<td>0.14</td>
</tr>
<tr>
<td>40 (LQC)</td>
<td>40.8 ± 2.8</td>
<td>0.07</td>
</tr>
<tr>
<td>200 (MQC)</td>
<td>197.8 ± 18.4</td>
<td>0.09</td>
</tr>
<tr>
<td>800 (UQC)</td>
<td>824.8 ± 21.1</td>
<td>0.02</td>
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</table>
EK, we needed to measure the concentration of each excipient in the bloodstream of the neonates. We chose a few excipients which are commonly used in these formulations (mainly parabens and ethanol). These were then analyzed in different laboratories within our consortium. Our laboratory was involved in developing and analyzing parabens from the DBS. This analytical data were then used to model EK for parabens which has been reported elsewhere [43].

Herein we report a simple, quantitative LC–MS/MS DBS parabens assay, which has been successfully developed, validated and applied to analyze neonatal samples. The circulating blood concentrations of parabens in neonates confirm systemic exposure to these compounds following administration of routine medicines. We report the first clinical study of systemic PB exposure in neonates. Only 40% of MPB and 14% PPB samples yielded the concentrations above the LOQ (20 ng/ml). This analytical data have now been subjected to population EK modeling. The clinical study of parabens among the children who received parabens in routinely administered medicines showed that the circulating concentrations of MPB are not sufficient to sustain concerns about long-term outcomes. PPB was below the limit of detection in half the children studied. This provides reassurance to clinicians, since the circulating concentrations are low and any metabolites are unlikely to have any endocrine disrupting effects [44].

Future perspective
DBS sampling and analysis has evolved fast in the last decade. With the increase of sensitivity of the new mass spectrometers, the DBS technology will be widely accepted by the academics and the industry as a very important sampling method. Those involved in animal research could reduce the number of animals used to a minimum, to obtain information from fewer animals or more information from the same number of animals by using the DBS technology. In our own work in the field

<table>
<thead>
<tr>
<th>Concentrations (ng/ml)</th>
<th>MPB</th>
<th>PPB</th>
<th>IS (5 ng/ml)</th>
</tr>
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<tbody>
<tr>
<td>20 (LOQ)</td>
<td>98.4</td>
<td>96.9</td>
<td>94.6</td>
</tr>
<tr>
<td>200 (MQC)</td>
<td>103.4</td>
<td>97.5</td>
<td></td>
</tr>
<tr>
<td>800 (UQC)</td>
<td>100.3</td>
<td>98.2</td>
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Table 3. Matrix effect.

Figure 3. Concentrations obtained from the patient sample analysis from each hospital site. (A) Concentrations above LOQ (20 ng/ml); (B) concentrations between 10 and 19 ng/ml. (C) Concentrations below limit of detection (10 ng/ml).
of pediatric research, this technology has been and will be very useful in future. To the specifics of our research in excipients – more excipients (commonly used in pediatric formulations) should be studied for their safe use. This could be done using existing expertise within Europe with the co-ordination with EMA and the pharmaceutical companies, by doing a multicenter trial.

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Financial & competing interests disclosure
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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research
The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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References
Papers of special note have been highlighted as:
1. of interest; ** of considerable interest
2. Acceptable daily intake of parabens.
3. This was one of the papers to initiate the ESNEE project.
4. Justification for the use of the DBS sampling.
5. One of the first publications to explain the concentration of methyl parabens in preterm infants.

Executive summary
• Measurement of methyl and propyl parabens concentrations from DBS by LC–MS/MS.
• DBS sampling was used to collect opportunistic blood samples (15 µl) from the neonates who have been administered parabens containing formulations.
• One hundred and ninety-six neonates were recruited from four UK and one Estonian site; a total of 927 DBS samples were available for analysis.
• This study addresses the systemic exposure of parabens to the neonatal population.

3. This was one of the papers to initiate the ESNEE project.


The first publication to demonstrate the use of DBS in infants.


Guidelines used for method validation.

- Guidelines used for method validation.


- Protocol followed for drug partitioning.


- Insource fragmentation of drug metabolites.


- Insource fragmentation of drug metabolites (glucuronides).


- Hematocrit and whole spot capture.


- Hematocrit and whole spot capture.


- Publication of the EK data based on the above analytical data.