Determination of levetiracetam in dried blood spot biology essay

Science, Biology

performance liquid chromatography tandem mass spectrometry 2Background: A simple LC-MS/MS method was developed and validated for the 7quantification of levetiracetam (LEV, Keppra®), a broad-spectrum antiepileptic drug 8(AED) in dried blood spots (DBS). LEV was simply extracted with methanol spiked with 9adenosine (ADE) as internal standard before LC-MS/MS analysis. The correlation 10between the DBS and plasma concentrations of LEV was also determined. 11Result: Linearity was from 0. 067-60 µg/mL for LEV in DBS samples. The intra- and inter- 12day accuracy and precision of the assay met validation acceptance criteria. DBS 13concentrations were well correlated to the plasma concentrations (R2= 0. 9399), 14asfraction of LEV bound to blood cells remains veryconstant (0. 466 ± 0. 041) over a wide 15concentration range. Conclusion: The study illustrated that DBS could be used as 16alternative matrixfor monitoringLEV in preclinical and clinical studies.

171819202122232425262728293031323334353 63Key Terms: 37Levetiracetam: 38Keppra®, (S)-α-ethyl-2-oxo-1-pyrrolidine acetamide, a second-generation, broad- 39spectrum anticonvulsant medication used to treat epilepsy 40Dried blood spots: 41Technique for collecting small whole blood samples, typically in 5–50 µl, on filter paper 42LC-MS/MS: 43Liquid chromatography -tandem mass spectrometry that combines two powerful 44techniques giving the chemical analyst the ability to analyze virtually any molecular 45species; including, thermally labile, non-volatile, and high molecular weight species 46Therapeutic drug monitoring: 47A branch of clinical chemistry and clinical pharmacology that specializes in the 48measurement of medication concentrations in blood 49Preclinical Study:

50A study to test a drug, a procedure, or another medical treatment in animals for support 51of the safety and suitabilityof the new treatment 52fBC: 53The fraction of analyte bound to blood cells, assumed constant, that can be used to 54predicate the plasma concentration of analyte 55565758459Levetiracetam(LEV, Keppra

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), (S)-α-ethyl-2-oxo-1-pyrrolidine acetamide, is a novel, 60broad-spectrum antiepileptic drug (AED)that is structurally unrelated to existing AEDs 61[1]. Thus far, its mechanism of action has not beenunderstood completely. It has been 62postulated to bind to the synaptic vesicle protein 2A (SV2A) in the brain [2, 3], thereby 63decreasing neurotransmission in epileptic circuits. LEV is rapidly and almost completely 64absorbed following oral administration (> 95%). In addition, It displays a linear 65pharmacokinetic profile over a wide range of therapeutic doses , insignificantplasma 66proteins binding (< 10%) as well asreadily and adequately crosses the blood brain 67barrier[4]. The drug’s minimal protein binding and non-involvement of hepatic 68metabolismincur no clinically significant drug interactions that confer upon it an 69exemplary safety profile. All these make it potentially useful for infants, young children 70and adult with epilepsy [5]. Nevertheless, despite its desirable features, the relationship 71between plasma concentrations of LEV and its clinical effect on different seizure types 72and intractability has not been clearly determined [6-9]. Hence, it has been 73recommended that therapeutic drug monitoring(TDM) of LEV be performed, 74particularly in renal-impaired patients[5, 6, 10-13], elderly and children, where half-life of 75the drug is extended [14] and

shortened [15], respectively. 76TDM as well as evaluation of systemic drug exposurein preclinical and clinical PK studies 77have traditionally relied onanalysing plasma samples. However, there are recent 78advocates on replacing this conventional sample matrix with dried blood spot(DBS) – 79whole blood blottedand dried on paper, asDBS is simpler to collect, manage, transport 80and store. Moreover, it reduces the blood volume required and the risk of HIV/AIDS and 81other infectionduring long term monitoring [16, 17]. Therefore, immense interest has 82been shown in the possible use of DBS as an alternative matrix to plasma with TDM of 83several drugs already using DBS as matrices [18-20]. Nevertheless, the use of DBS 84monitoring of LEV has not been established, despiteseveral analytical methods reported 85for the measurement of LEV in plasma and blood)[21]using gas chromatography (GC) 86with nitrogen-phosphorus detection [21], GC-mass spectrometry (GC-MS) [22], 87microemulsionelectrokinetic chromatography [23] andnumerous high performance 88liquid chromatography (HPLC) techniques [24-27]. There are few reportedmethods [11, 8913, 21, 22] for the analysis of LEV using LC-MS/MS, two of which [13, 21] employed solid 90phase extraction (SPE), an elaborate and tiresome procedure. 91Therefore, the objective of this study was to develop and validate a rapid, reliable, 92selective, sensitive, and accurate LC-MS/MS method to quantify LEV in preclinical study, 935to assess DBS suitability by fBCand relationship between the LEV levels in DBS and 94plasmaas an alternative matrix for TDM of LEV. 95Experimental 96Chemicals and reagents 97Levetiracetam (LEV) was purchased from Cell Molecular Pharmaceutical R&D Co., Ltd. 98(Xi’an, China) and the internal

standard (IS), adenosine from Sigma-Aldrich Co. 99(Singapore). Water was purified through the use of MilliporeTMDirect-Q 3 UV Water 100Purification System (Singapore), while other chemicals and reagents were of analytical 101grade and solvents were of HPLC grade. 102Instrumentation and Chromatographic Conditions 103TheLC-ESI-MS/MS system used was composed of a modelShimadzu UFLC system 104(Shimadzu Scientific Instruments, Columbia, MD) coupled to a Q TrapTM3200 hybrid 105triple quadrupole linear ion trap mass spectrometer(Applied Biosystems/MDS Sciex, 106Concord, Ontario, Canada). Data processing was performed with AnalystTM1. 4. 2 107software package (Applied Biosystems, MA., USA). 108109Chromatographic separation was performed on aZorbaxEclipse Plus C18 column (2. 1 x 110100 mm, I. D., 3. 5 µm, Agilent Technologies, Palo Alto, CA, USA) with a Security Guard 111Cartridge (3. 0 X 4 mm, Agilent Technologies, Palo Alto, CA, USA). The column 112temperature was ambient, the flow rate was 0. 25 mL/min and the injection volume was 1132 µL. The mobile phase A was 0. 1% formic acid (FA) in MilliporeTMwater and mobile 114phase B was 0. 1% FA in methanol. The mobile phase gradient was as follows: initial 10% 115B linear increased to 98% B in 0. 3 min and held for2. 6 min, finally dropped to 10% B 116within 0. 2min and equilibrated for 1. 9 min. The total run time was 5min. 117118The mass spectrometer was operated using ESI sourcein the positive ion detection 119mode for LEV determination. Acquisition was performed in multiple reaction monitoring 120(MRM) mode using m/z171→126 and m/z268→136 for LEV and IS, respectively. The 121optimized instrument parameters for monitoring theanalytes by mass spectrometry are 122as follow: source temperature

(TEM), 450°C; turbo spray voltage (IS), 5400 V; curtain gas 1236(CUR), 10 psi; Nebulizing gas (GS1), 40 psi; turbo ion spray gas (GS3), 40 psi; collision gas 124(CAD), medium; and dwell time 200 ms. 125Stock standards, calibration standard and quality control samples 126Primary stock solution of LEV was prepared by dissolving an accurately weighted 127amount in methanol: water (1: 1) to yield 10 mg/mL. The stock solution was then further 128diluted in methanol: water (1: 1) to give working standard solutions with LEV 129concentrations of 1, 3, 30, 100, 300, 720 and 900µg/mL. DBScalibration standards of LEV 130were prepared by spiking 42µL fresh blank SD rat blood with 3 µL of a standard LEV 131working solution, producing LEV blood concentrationof 0. 067, 0. 2, 2, 6. 67, 20, 48 and 60 132µg/mL. Aliquots (15 µl) of the calibration standards samples were spotted onto punched 133out discs of Guthrie DBS paper (U. K. Neonatal Screening Laboratories Network- 134Whatman 903

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) and allowed to dry overnight at room temperature. The DBS calibration 135standards and QC samples were used immediately uponcompletion of drying. The 136quality control (QC) samples were similarly prepared in blank blood at the 137concentrations of 0. 2, 6. 67 and 48µg/mL. The stock solution of the IS wasprepared in 138methanol: water (1: 1) in 1mg/mL and were further diluted in methanol to yield a 139working stand solution of 3 µg/mL. 140141For assay of LEV in plasma samples, the working standard solutions with concentration 142of 0. 05, 0. 15, 1. 5, 5, 15, 36 and 45 µg/mL of LEV in methanol were prepared. Calibration 143standards of LEV were

prepared by spiking 20µL fresh blank SD rat plasma with 20µL of a 144standard LEV working solution, producing calibration samples as equivalent to the 145plasma concentrations of 0. 05, 0. 15, 1. 5, 5. 0, 15, 36 and 45 µg/mL. The quality control 146(QC) samples were similarly prepared in blank plasma at the concentrations of 0. 15, 5 147and 36 µg/mL. The stock solution of the IS were prepared in methanol: water (1: 1) in 1481mg/mL and were further diluted in methanol to yield a working stand solution of 6 149µg/mL. 150151All the solutions were stored at -20°C and brought to room temperature before use. 152Pharmacokinetic (PK) study 153The in vivostudy was carried out according to the " Guidelineson the Care and Use of 154Animals for Scientific Purposes" (National AdvisoryCommittee for Laboratory Animal 155Research, Singapore, 2004). The animal handling procedures were reviewed and 1567approved by the Institutional Animal Care and Use Committee of the National University 157of Singapore (NUS). 158159Six male Sprague-Dawley rats, weighing 290 to 320g (9 weeks old) were purchased from 160Comparative Medicine, Centre for Life Sciences of NUS. The rats were kept at a specific 161pathogen-free animal facility (24°C, 60% relative humidity) and maintained on a 12-hour 162light/dark cycle with free access to a rodent autoclavable diet and water. At 24 hours 163before the study, the animals were anaesthetized, and a polyethylene tube was inserted 164into the right jugular vein for blood sampling. Following 1 day of post-surgical recovery, 165catheter patency was ensured prior to the intravenous administration of LEV constituted 166in 0. 9% saline (25. 0 mg/ml) and dosed at 40 mg/kg. Serial blood samples (approximately 167300 µL) from each rat were collected at 0, 5, 15, 30, 45min and 1, 2, 4, 6, 8, 24h.

All 168samples were placed into heparinized tubes. To prepare a DBS sample, 15 µL of blood 169was spotted onto the disc (6 mm) punched out from the Guthrie paperand allowed to 170dry overnight at room temperature prior to storage at -80°C. At the same time the rest 171of the blood taken was centrifuged and the plasma was stored and afterwards used to 172determine the plasma concentration of the same drugwith the developed and validated 173LC-MS/MS assay described in previous studies [5, 13, 22]. The assay was further validated 174by comparing the LEV levels in DBS and plasma samples collected from Sprague Dawley 175rats. Correlation between the DBS and plasma levelsand fraction of LEV bound to blood 176cells (fBC) were computed. 177Sample preparation 178Prior to assay, calibration standards, QC samples and frozen rat samples were thawed at 179ambient temperature. For DBS samples, extraction was carried out by adding 200 µL of 180methanol containing the IS (3 µg/mL) to each Eppendorf tube containing a DBS 181specimen. The samples were vortexed for 60 s, then sonicated for 5 min, followed by 182centrifugation at 10 000 rpm (4°C) for 5 min. 150 µl of the clear supernatant was 183transferred and 2 µL injected into the LC-MS/MS system. 184185For assay of LEV in the plasma sample, protein precipitation was carried out by mixing 18620-µL aliquot of plasma sample and 20 µL methanol: water (1: 1) with 160 µL of 187methanol containing the IS (6 µg/mL). The samples were vortexed for 30s, followed by 188centrifugation at 10, 000 rpm for 10min. 120µL of the clear supernatant was transferred 1898into the 96-wells plate at 4°C. Then, a 2-µL aliquot was injected into the HPLC-MS/MS 190system for analysis. 191192Method Validation 193Assay validation was performed according to the

FDAguideline[23]. Selectivity was 194assessed by comparing six individuals DBS blank samples with those obtained from 195spiking blank blood with LEV at the lower limit of quantification (LLOQ). Quantitative 196analysis of LEV in DBS and plasma samples was performed using IS method. Calibrations 197were built from peak area ratios of analyte vs. IS and linearity was assessed by weighted 198(1/x2) least-squares analysis. During validation, the calibration curves were defined on 199three different days based upon assays of the spiked duplicate samples, and QCs were 200determined in five replicates on the same day. Accuracy and precision were also 201assessed by determining QC samples at three concentration levels on three validation 202days. The accuracy was expressed by relative errors(RE) and precision by relative 203standard deviation (RSD). 204The lower limit of quantification (LLOQ), defined as the lowest concentration at which 205both precision and accuracy were less than or equalto 20%, were evaluated by 206analyzing samples which were prepared in five replicates. 207The extraction recoveries of LEV were determined bycomparing the peak areas of QC 208samples to those of LEV added post-extraction in blank DBS sample. To evaluate the 209matrix effect, a post-extraction addition method was also utilized. 210The analyte stability in DBS samples under processing was evaluated at the 211concentrations of QC samples. The bench-top stability was determined DBS samples at 212ambient temperature for 24 h. The freeze-thaw stability was studied after three 213successive freeze-thaw cycles at -80°C. Similar experiments were performed for long- 214term stability evaluation after 30 days storage at -80°C. Stability was also assessed after 215processed DBS samples in the instrument autosamplerat

approximately4°C. The results 216were compared with those QC samples freshly prepared. 2172189Results and discussion 219Method validation 220Selectivity 221Potential interference from endogenous compounds was investigated by analyzing rat 222plasma of six different subjects. FIGURE1 shows the typical chromatograms of a blank, a 223spiked DBS sample with LEV at lower limit of quantification (LLOQ) and the internal 224standard. No significant interference or ion suppression from endogenous substances 225was observed at the retention time of the analyte and IS. The retention times were 226approximately 2. 64 min and 2. 82min for IS and LEV, respectively. 227Linearity of calibration curves 228The linear regressions of the peak area ratios versus concentration were fitted over the 229concentration range of 0. 067-60 µg/mL for LEV in DBS samples. The typical equation of 230the calibration curves was as follows: y= 2. 3e3x+0. 00434, r= 0. 9969 Where y represents 231the peak ratio of analyte to IS and x represents the concentration of the analytes in 232plasma. The correlation coefficient (r) exceeded 0. 99, showing a good linearity among 233the concentration range. 234Precision and accuracy, LLOQ, Extraction recovery and matrix effect 235The intra- and inter-day precision and accuracy forLEV in QC and LLOQ samples are 236given inTABLE1. The lower limit of quantification (LLOQ) was 0. 067µg/mL for LEV. The 237intra and inter-day RE and RSD were less than 7%at LLOQ level. For all the QC samples, 238the intra and inter-day precision was below 9% and the RE was from 2. 8% to 7. 5%. 239Therefore, the method presented acceptable accuracyand precision. 240The mean extraction recoveries were also shown in TABLE2. The RSD was less than 6% for 241all recoveries throughout the entire standard

concentration ranges, showing good 242consistency. The matrix effects calculated were in the range of93% to 101%. Therefore, 243ion suppression or enhancement from rat blood was negligible under the current 244conditions. 245Stability 246DBS offers a simpler and better method for storage as it allows the samples to bemore 247stable at room temperature. LEV in DBS samples proved to be stable at room 24810temperature, even for 24h (REs≤10%). Stability was also assessed using the processed 249DBS samples in the instrument autosampler at approximately 4°C for 8h. The analyte 250wasfound to bestable with percentage of LEV remaining at≥90%. Similar experimental 251results were obtained for the three successive freeze-thaw cycles at -80°C andthe long- 252term stability evaluation after 30 days storage at -80°C. 253Application to PK study in the Sprague-Dawley rats 254PK study 255This validated method was successfully applied to the PK studies after intravenous 256administration of 40 mg/kg LEV in Sprague-Dawley rats. Their concentration-time 257profiles are shown in FIGURE2. Generally they showed linear PK profile, which conforms 258with those found in other studies[11, 21] 259Correlation between DBS and Plasma 260To determine a quantitative relationship between the LEV levels in DBS and plasma 261samples, the corresponding DBS and plasma levels determined by the validated LC- 262MS/MS method were compared (FIGURE3). The good correlation were shown(R2263= 0. 9399) with the slope values higher than 1, whichindicated higher concentration 264values determined from plasma samples. The DBS concentrations were 33% (SD 7. 1%) 265lower than the corresponding plasma concentrations. Since LEV binds minimally to 266plasma proteins (< 10%)[4], the total plasma concentration of the drug

will be 267approximately equal to its unbound plasma concentration. As DBS concentration is a 268measure of the whole blood concentration, the observation of the consistently lower 269LEV DBS concentrations is probablyas a resultof thelower concentration of LEV in whole 270blood. Indeed, erythrocytes often serve to dilute the drug in whole blood compared to 271plasma due to the extra volume provided by red blood cell according to the literature 272[24]. This would explain the lowerLEV concentrationsin DBS samples and blood samples 273when compared to the plasma concentration found in this study and other previous 274study[21]. 275Hematocrit is the most important determinant of whole blood viscosity. It impacts the 276flux and diffusion properties of blood spotted ontoGuthrie paper, and subsequently, the 277concentration of each sample [20, 25, 26]. This haematocrit effect was minimized in this 278study, asexperimental inbred animals were used and they were expected to have fairly 27911uniform haematocrit value. Li et al.[24] modified a formula proposedby Eyles et 280al.[27]and applied it to correlate the DBS analyte concentration, DBS[analyte]with 281theplasma analyte concentration, plasma[analyte]. The formula incorporates both 282haematocrit and fraction of an analyte bound to blood cells(fBC): 283(DBS[analyte]/[1-haematocrit])×(1- fBC) = plasma[analyte]284fBCwas originally assumed to be constant in this formula. As mentioned earlier, the 285haematocrit should be fairly constant in our homogenous population of inbred 286rats. Weherein chose to affix the rat haematocrit value at40%, as indicated by a previous 287study[28]. With the fixed hematocrit value, the fBCvalues were then computed. It was 288found that the fBCvalues remained very constant throughout the wide

concentration 289range, at0. 466±0. 041 (mean +SD), which supported the widely accepted assumption of 290constantfBC, thus further validating our study. 291While we are accustomed to using plasma concentrations for TDM, it has been 292recommended that the whole blood drug concentrationbe used since it gives a better 293prediction of the therapeutic effectthan the plasmadrug concentration [18]. Clinically, 294more variation in haematocrit values is anticipatedin patients, thusthe hematocrit levels 295should be included when interpreting the correlation of LEV levels in DBS to plasma. 296Constant fBCvalues of LEV wasfound in this study. Thus, when LEV DBS levels are 297converted to their corresponding plasma levels, only thehematocrit valuesneed to be 298included in the conversion. If the hematocrit values are anticipated to be fairly constant 299in the study subjects, a population average value could be adopted in the calculation. 300301Conclusion 302This study describes an alternative determination of LEV from DBS samples using LC-ESI- 303MS/MS method. It has proved to be rapid, sensitive, selective, accurate and precise and 304gave reliable results. The DBS concentrations were also found to be well correlated to 305their plasma concentrations (R2= 0. 9399). In addition, fBCremained constant over a wide 306range of drug concentrations. Thus, it is noteworthy that when converting the DBS 307concentrations to the plasma concentrations, the hematocrit levels need to be 308considered in patients with anticipated fluctuationin the levels. Our findings illustrated 309that DBS could be used as a matrix for quantification of LEV for PK studies and TDM. 310311Future perspective 31212The use of dried blood spots (DBS) as a sampling technique was introduced in the early 3131960s. It has been routinely

carried out on infantsand more recently, for the TDM of 314several drugs. DBS offers a more patient-friendly and convenient alternative to 315conventional venous blood sampling. DBS-LC-MS/MS, involving the use of highly 316sensitive LC-MS/MS system to quantify DBS samples, is an emerging topic in the 317pharmaceutical community and is expected to play anincreasing role in drug discovery 318and TDM. Our study demonstrates the feasibility of such approach for quantifying LEV 319by establishing the excellent correlation between DBS levels and conventional plasma 320levels. This finding has opened up new avenues for the bioanalysis of LEV and will be 321especially useful in the TDM of this anti-epilepticdrug. 322323Executive summary 324The first paper to report a simple LC-MS/MS method for quantification of 325levetiracetam in dried blood spot with a good LLOQ of 0. 07 µg/mL. 326The method developed was fully validated in terms of linearity, selectivity, accuracy, 327precision and stability and proved to be robust andreliable. It was also successfully 328applied to a preclinical study. 329Good correlation between the LEV levels in DBS and plasma samples were found 330(R2= 0. 9399). In addition, fBC(0. 466 ±0. 041; mean ± SD) remained very constant over a 331wide drug concentration range. 332The results support the use of DBS as a matrix for quantification of LEV for 333pharmacokinetic studies and therapeutic drug monitoring. 33433533613337338