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DBS System

GERSTEL AppNote

Pharmacokinetic study on carbamazepine and gabapentin employing HemaXis DB, a novel device for convenient whole blood self-sampling, and the GERSTEL Dried Blood Spot Autosampler (DBS-A) coupled online to LC-MS/MS

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Abstract
This application note presents the automated determination of carbamazepine, its metabolite carbamazepine-10,11-epoxide and gabapentin in dried blood spots (DBS) using the GERSTEL Dried Blood Spot Autosampler (DBS-A) coupled online to LC-MS/MS. The analysis method was employed to demonstrate the feasibility of pharmacokinetic studies with this type of instrumentation. Concentration vs. time curves were recorded for the analytes in real dried blood samples from a volunteer person after application of a single oral dose of each drug. Data generated were in agreement with pharmacokinetic data from scientific literature.

In the context of this application a novel DBS sampling device - HemaXis DB - for simplified collection of volumetric DBS by minimally trained users was employed. The sampling system proved reliable and delivered good precision. Relative standard deviations for repeat analyses after sampling of real blood with HemaXis DB were between 3.1 and 11% for the target compounds. Manual pipetting of spiked whole blood onto DBS cards and subsequent analysis resulted in similar relative standard deviations. Limits of quantification (LOQ) were estimated as 8 ng/mL for carbamazepine, 4 ng/mL for carbamazepine-10,11-epoxide, the major carbamazepine metabolite, and 2 ng/mL for gabapentin, respectively.

The GERSTEL Dried Blood Spot Autosampler (DBS-A) and the HemaXis DB device were found to be valuable tools for pharmacokinetic studies, helping to reduce cost of sampling and sample shipment while improving data quality since repeat or more frequent blood sampling is feasible without the need for healthcare professionals to perform the sampling.

Introduction
Gabapentin is a medication used to treat epilepsy, neuropathic pain and other medical conditions. Carbamazepine is an anticonvulsant drug which is employed against epilepsy. Furthermore it can be administered in certain cases of mental disorders. Both drugs are essential for multiple different therapies and are commonly used worldwide [1].

According to the International Union of Pure and Applied Chemistry (IUPAC) pharmacokinetics is 1. the “Process of the uptake of drugs by the body, the biotransformation they undergo, the distribution of the drugs and their metabolites in the tissues, and the elimination of the drugs and their metabolites from the body over a period of time”. 2. “Study of such processes” [2]. Pharmacokinetic studies are one mandatory step in the drug approval process. Studies with large numbers of probands and samples need to be conducted. Besides urine, usually plasma or blood

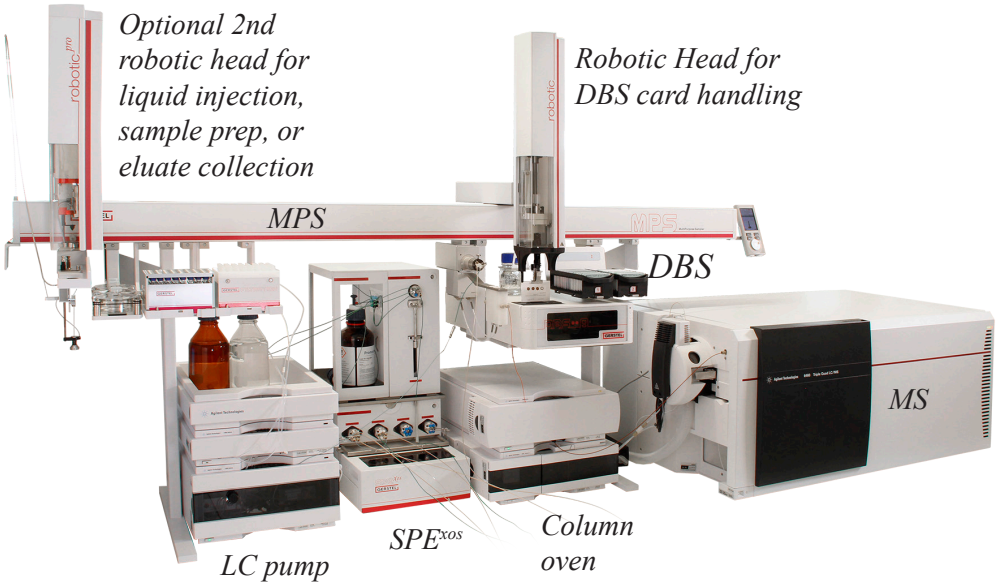


Figure 2. GERSTEL DBS-A for automated dried blood spot analysis coupled online to an LC-MS/MS sytem. The MPS delivers DBS cards into the desorption module (DBS), SPE is used for cleanup of the eluate and introduction to LC-MS/MS. The optional 2nd Head of the MPS is for sample preparation, liquid injection or eluate collection.

respectively are the matrices of choice. Samples are taken at different time points over a couple of days; sampling must be performed by medically trained personnel and the samples must be refrigerated during transport to the laboratory. In the laboratory, blood samples are finally centrifuged to obtain blood plasma, which can be extracted and analyzed.

The dried blood spot sample format can be an alternative to plasma in such studies in order to reduce cost for sampling, sample shipping and handling, and to improve data quality [3]. A few drops of blood (e.g. 5-20 µL) from a finger prick dried on special cellulose cards are sufficient for a wide range of applications. Self-sampling can be done by the proband at home without the presence of medically trained personnel. After drying, the samples can be shipped to the laboratory without being refrigerated and without biohazard labeling. Furthermore, handling in the laboratory is easier and less hazardous compared to liquid whole blood or plasma samples.

Introduced by Guthrie in the 1960´s for neonatal screening for inborn diseases [4] the dried blood spot technique spread around the world in the following decades. In the 2000s with the advent of sensitive LC- and GC-MS instrumentation it gained attention also in other areas of bioanalysis, such as pharmacology, forensic toxicology, clinical- and anti-doping analysis [3,5-7].

In conventional manual sample preparation a disc (e.g. 3 mm diameter) is punched out of the dried blood spot for subsequent solvent extraction of the analytes, for example, using methanol. The extract can undergo further cleanup or can be analyzed directly. The process of punching and extracting is rather tedious especially for larger sample numbers. For this reason, automation of sample preparation was pursued and Déglon et al. reported the first automated DBS analysis by flow through desorption [8]. SparkHolland further improved this technique and together with GERSTEL integrated it into the Dried Blood Spot Autosampler (DBS-A). The blood spot is clamped leak tight into a flow path and analytes are desorbed by a solvent flow through it (Flow Through Desorption™). The desorbed analytes can be introduced online to an LC/MS system after solid phase extraction (SPE) cleanup or can be collected in vials for optional further processing and subsequent injection. In figure 1, a schematic is shown that illustrates the working principle of the DBS-A and in figure 2, a hardware setup example is shown. The DBS-A has recently been utilized for anti-doping analysis, an emerging field of application for DBS [9-11]

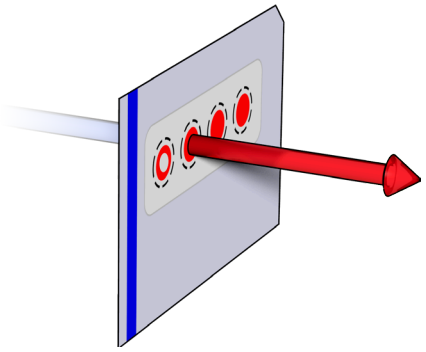


Figure 1. Principle of Flow Through Desorption™ (FTD) for automated dried blood spot analysis.

In order to simplify sampling and to sample blood spot volumes with high accuracy and precision, enabling full spot analysis, HemaXis DB devices were used (figure 3, DBS System SA, Gland, Switzerland). The goal of our work was to show the feasibility of conducting pharmacokinetic studies based on dried blood spot samples taken with help of HemaXis DB and analyzed by the DBS-A coupled online to an LC-MS/MS system.

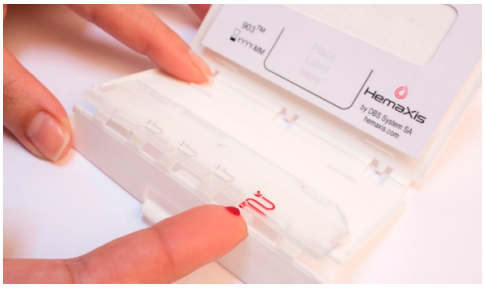


Figure 3. HemaXis DB10 device for convenient exact volume blood spot sampling, secure sample shipment and storage.

Experimental

Instrumentation. The GERSTEL Dried Blood Spot Autosampler (DBS-A) consists of a MultiPurpose Sampler (MPS), which delivers cards to the DBS desorption module alongside an online Solid Phase Extraction system (SPE^{xos}) for extract cleanup before transfer to the LC-MS/MS. In this case the MPS was configured with a second head enabling further sample preparation steps, as well as liquid injection into the LC/MS system via a valve for example for method development purposes. The completely automated DBS workflow consists of the following steps:

- The Automated Cartridge Exchanger (ACE) of the SPE^{xos} module inserts a 1 cm SPE cartridge into the cartridge clamp.
- Cartridge conditioning solvent is delivered by the High Pressure Dispenser Module (HPD) of the SPE^{xos}.
- The MPS card gripper picks up a DBS card from the rack and inserts it into the DBS module.
- A photograph of the card is taken in order to document the quality of the spot, localize it, and optionally to read the barcode. In case of invisible spots, e.g. plasma or urine, the dotted circle on the card is located.
- The blood spot is sealed leak tight into the flow path at the determined position by a set of clamps of 2, 4, 6 or 8 mm diameter, depending on requirements.
- Optionally the internal standard loop (20 µL) is filled by the built-in pump.
- Analytes are desorbed from the dried blood spot in flow through mode by one or more solvents delivered by the HPD and trapped on the SPE cartridge. An internal standard can be added automatically through an injection loop. The desorption solvent can be pre-heated by the hot cap unit as needed.
- Flow paths are dried by a built-in compressor, the card is released and as an option re-sealed at an empty position on the card to clean the clamps using one or more solvents.
- After desorption, a photo of the card is taken for QC and documentation purposes.
- Optionally the SPE cartridge is washed.
- The LC eluent is directed through the cartridge and elutes the analytes into the LC-MS/MS system. We used a SPExos system suitable for 300 bar; a 1000 bar system is available for UPLC coupling.
- When the elution step has been completed, the cartridge is removed from the LC flow path and exchanged (optionally) in order to prevent sample to sample analyte carryover.

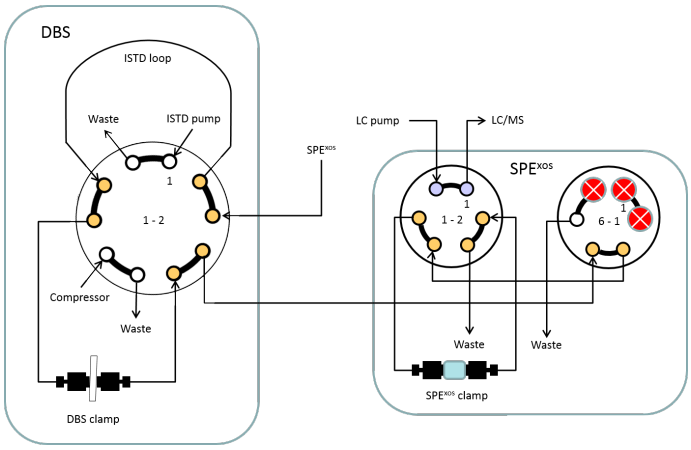


Figure 4. Flow diagram of the DBS-A used in online mode.

The described online setup used in this application enables a 100% transfer of the eluted analytes to the LC-MS/MS system making the analysis highly sensitive compared to when only an aliquot is injected. Alternatively, in workstation mode, the eluate from either the blood spot or the SPE cartridge can be collected in vials or well plates allowing further

automated sample processing steps such as evaporation and reconstitution, derivatization, filtration or liquid/liquid extraction to be performed. Finally the prepared samples can be injected to an LC/MS or GC/MS for analysis.

The LC-MS/MS system used was an Agilent 1260 LC coupled to an Agilent 6460 triple quadrupole mass spectrometer. The separation column used was an Agilent Poroshell 120 EC-C18, 3 x 50 mm, 2.7 µm.

Materials, Chemicals and Standards

Solvents were of LC/MS grade from Carl Roth. Salts were of p.a. grade from Merck (Sigma-Aldrich). Pig blood serving as blank matrix for calibration was purchased from a local butcher and stabilized with 2 g di-sodium-ethylenediaminetetraacetate (EDTA) directly upon receipt.

Analyte standards and internal standards were purchased from Merck as follows: carbamazepine (neat, 1 g), carbamazepine-10,11-epoxide solution (1000 µg/mL in methanol), gabapentin (neat, 1 g), carbamazepine-D10 solution (100 µg/mL in methanol) and gabapentin-D10 solution (100 µg/mL in methanol).

For capillary blood sampling HemaXis DB whole blood collection devices (DBS System) were used. HemaXis DB incorporates a microfluidic chip for accurate and precise volume sampling, a standard cellulose card (Whatman 903 Protein Saver) and a protective polymer envelope. The credit card sized device provides a practical solution for whole blood collection from a fingertip that can be performed anywhere at any time. It allows blood samples to be transported and stored at room temperature in a dry format. Full spot analysis enables results without hematocrit bias. In this study HemaXis DB5 for 5 µL blood sampling was used, but currently only the HemaXis DB10 10 µL version is commercially available.

Preparation of Calibration Standards and Internal Standard Solutions

Single stock solutions in water with a concentration of 1 mg/mL were prepared from all neat analytes. These stock solutions and the purchased reference standards were combined and diluted with water to produce three different working standards of 200, 20 and 2 µg/mL per analyte. Small volumes of the working standards were combined with large volumes of pig blood resulting in blood-based calibration standards with analyte concentrations between 10 and 4,000 ng/mL. Aliquots of 5 µL each of these calibration standards were spiked on 903 Protein Saver DBS cards, as included in the HemaXis DB device, using an Eppendorf pipette. Cards were dried at room temperature for a couple of hours and then directly used for calibration or stored for some days in a refrigerator at 8°C in a plastic bag containing a silica gel drying bag (N077.2, Carl Roth). The described workflow resulted in absolute amounts of 0.05 to 20 ng per blood spot for calibration. Cards for precision and LOQ measurements were prepared in a similar way.

Two separate internal standard (ISTD) solutions were prepared from the purchased deuterated gabapentin and carbamazepine reference standards. 50 µL aliquots of the individual reference standards were filled up to 100 mL in separate volumetric flasks using 2 mM ammonium formate solution (pH 7) for gabapentin and water for carbamazepine. The final concentration was 0.05 µg/mL. Based on the 20 µL ISTD loop volume, this resulted in an absolute amount of 1 ng per ISTD compound on column.

Design of the Experiment and Sampling

A single dose of a 200 mg carbamazepine retard tablet was administered to a female, healthy volunteer. In a second experimental setup the same volunteer took a single dose of a 300 mg gabapentin tablet. The volunteer had taken both medications previously for medical reasons, but had not ingested these for a prolonged period of time so that the initial blood concentration was expected to be zero.

Capillary blood was self-sampled by the volunteer at defined time points using a HemaXis DB device. For carbamazepine duplicate samples were taken at each time point, for gabapentin just one. For two time points, five-fold sampling was performed to document the precision of the sampling procedure. No sampling was conducted during the night. The study for carbamezepine was concluded 168 h (seven days) and for gabapentin 36 h (one and a half days) after the substance had been administered.

Blood samples were taken from the little finger or ring finger. The skin was cleaned using a hand disinfectant. An 18G safety lancet with a penetration depth of 1.8 mm (85.1017, Sarstedt) was used for the finger prick and the finger was squeezed lightly to bring out the first drop of blood, which was removed by a clean tissue. The next drop formed was brought in touch with the microfluidic unit of the HemaXis DB device until the capillary was filled completely. Afterwards HemaXis was closed causing the blood to be transferred quantitatively onto the blood spot card. After sampling,

the device was re-opened to enable drying of the blood. Until it could be analyzed, the HemaXis DB device loaded with sample was stored for several days at 8°C inside a plastic bag containing a silica gel drying bag (N077.2, Carl Roth).

Automated DBS Workflow
The DBS-A was used in online mode, directly coupled to the analysis system. This means that the operator places the DBS cards into the autosampler rack after which the complete analysis is performed automatically including sample preparation and LC-MS/MS analysis. Separate methods were used for carbamazepine and gabapentin, the method parameters are listed below:

Carbamazepine and Carbamazepine-10,11-epoxide

SPE^{xos}/DBS-A
Cartridge: HySphere C18 HD, 2 x 10 mm, 7 µm
Cartridge conditioning: 2 mL acetonitrile, 2 mL 0.1% formic acid @ 5 mL/min
Clamp diameter: 6 mm, full spot sampling
Blood spot desorption: 2 mL water including 20 µL internal standard @ 1 mL/min and 80°C hot cap temperature
Clamp washing: 2 mL 0.1% formic acid, 2 mL acetonitrile, 2 mL water @ 5 mL/min
Cartridge washing: 1 mL 0.1% formic acid @ 2 mL/min
Elution: LC gradient for 3.4 min

LC
Column: Poroshell 120 EC-C18, 3 x 50 mm, 2.7 µm (Agilent Technologies)
Eluent A: 0.1% formic acid
Eluent B: acetonitrile
0 min: 95% A, 5% B
5 min: 40% A, 60% B
5.5 min: 10% A, 90% B
6.5 min: 10% A, 90% B
7.5 min: 95% A, 5% B
Flow: 0.5 mL/min
Temperature: 30°C

MS
Gas Temp: 300°C
Gas Flow: 9 L/min
Nebulizer: 45 psi
Sheath Gas Temp: 250°C
Sheath Gas Flow: 11 L/min
Capillary Voltage: 2000 V
Nozzle Voltage: 2000 V
MRM: See table 1

Gabapentin

SPE^{xos}/DBS-A
Cartridge: HySphere C18 EC-SE, 2 x 10 mm, 8 µm
Cartridge conditioning: 4 mL water, 4 mL acetonitrile, 2 mL 2 mM ammonium formate @ 5 mL/min
Clamp diameter: 6 mm, full spot sampling
Blood spot desorption: 0.5 mL 2 mM ammonium formate including 20 µL internal standard @ 0.5 mL/min and 90°C hot cap temperature
Clamp washing: 2 mL 2 mM ammonium formate, 2 mL acetonitrile, 2 mL 2 mM ammonium formate @ 5 mL/min
Cartridge washing: None
Elution: LC gradient for 7 min

LC
Column: Poroshell 120 EC-C18, 3 x 50 mm, 2.7 µm (Agilent Technologies)
Eluent A: 2 mM ammonium formate
Eluent B: acetonitrile
0 min: 95% A, 5% B
8 min: 10% A, 90% B
9 min: 10% A, 90% B
10 min: 95% A, 5% B
Flow: 0.5 mL/min
Temperature: 30°C

MS
Gas Temp: 300°C
Gas Flow: 9 L/min
Nebulizer: 45 psi
Sheath Gas Temp: 250°C
Sheath Gas Flow: 11 L/min
Capillary Voltage: 2000 V
Nozzle Voltage: 2000 V
MRM: See table 1

Compound	m/z Precursor Ion	m/z Product Ion	Dwell Time [ms]	Fragmentor Voltage [V]	Collision Energy [V]	Cell Accelerator Voltage [V]	Polarity
Carbamazepine	236.9	194	20	140	15	5	Positive
Carbamazepine	236.9	179	20	140	40	5	Positive
Carbamazepine D_{10}	247	204.1	20	140	20	1	Positive
Carbamazepine D_{10}	247	202	20	140	40	1	Positive
Carbamazepine-10,11-epoxide	252.9	236	20	110	5	6	Positive
Carbamazepine-10,11-epoxide	252.9	180	20	110	25	5	Positive
Gabapentin	172	154	20	110	10	4	Positive
Gabapentin	172	137	20	110	15	4	Positive
Gabapentin D_{10}	182	164.1	20	110	10	4	Positive
Gabapentin D_{10}	182	147.1	20	110	15	5	Positive

Table 1. MRM parameters for carbamazepine, its metabolite carbamazepine-10,11-epoxide and gabapentin.

Results and Discussion

Since gabapentin is very polar (log K_{ow} = -1.1) it is only very briefly retained on the SPE cartridge and cannot be combined with carbamazepine in one online DBS-A run. Two separate automated methods were therefore developed, one for the determination of carbamazepine/carbamazepine-10,11-epoxide and one for gabapentin in dried blood spots. Both methods were based on using the DBS-A in online mode, directly coupled to the LC-MS/MS system. Figure 5 shows a typical blood spot card from the HemaXis device after analysis of spot 1.

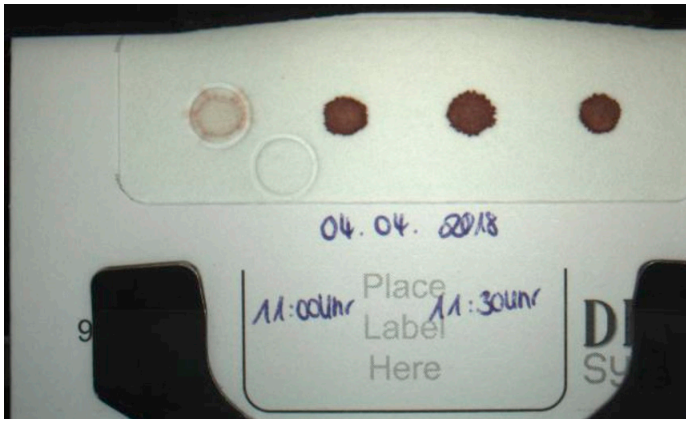


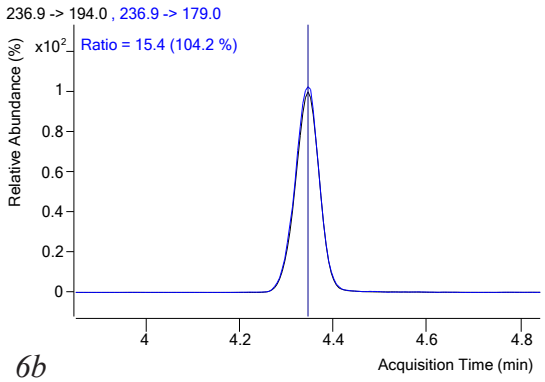
Figure 5. Picture taken by the built-in DBS-A camera showing a typical HemaXis DB blood spot card after analysis of spot 1.

In contrast to the workstation mode, in which only an aliquot of the analytes from the blood spot is transferred to the LC-MS/MS, the online mode offers better sensitivity since analytes from the whole blood spot are transferred quantitatively to the LC-MS/MS. The turnaround time is 14 min for the carbamazepine and 18 min for the gabapentin method. There is potential for further analysis time reduction, but that goal was not pursued in this study.

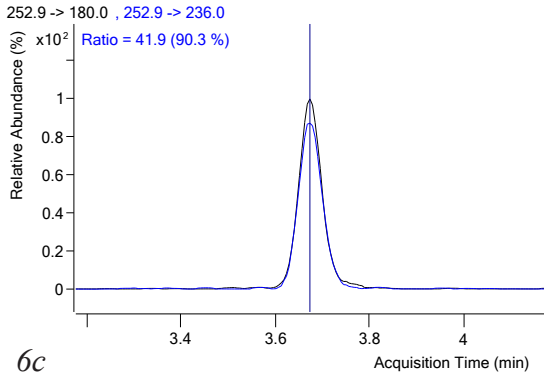
The LC-MS/MS method was developed by injecting liquid standards of the substances via the LC valve included in the MPS setup (see figure 2). A screening for the best suited SPE sorbent to retain the compounds of interest after elution from the blood spot was performed. Two different silica based C18 materials were identified as optimal sorbents. SPExos and DBS parameters were optimized further utilizing DBS cards loaded with 5 µL of spiked pig blood as samples. SPExos cartridges can be re-used five times for this application without biasing analysis results.

Analyte peaks are well separated from potential coeluting compounds as can be seen in figures 6a to c. Calibration ranges, as well as estimated limits of quantification (LOQs) based on an S/N >10 for quantifier and qualifier MRM and, finally, precision data are all summarized in table 2. The complete transfer of eluted analytes into the LC-MS/MS system in online DBS-A mode enables single digit ng/mL LOQs from only 5 µL of whole blood. Precision is very good for all analytes, relative standard deviations ranged from 3.1 to 11%. Using HemaXis DB for blood sampling resulted in precision similar to what is achieved when spotting spiked blood using an Eppendorf pipette.

6a



6b



6c

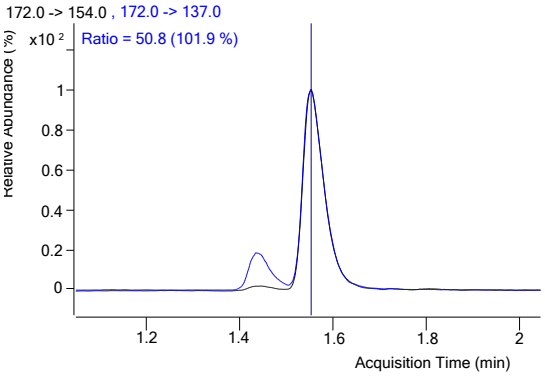


Figure 6a-c. Typical quantifier and qualifier analyte peaks from real test person´s DBS samples. 6a carbamazepine 324 ng/mL, 6b carbamazepine-10,11-epoxide 15.2 ng/mL, 6c gabapentin 65.6 ng/mL.

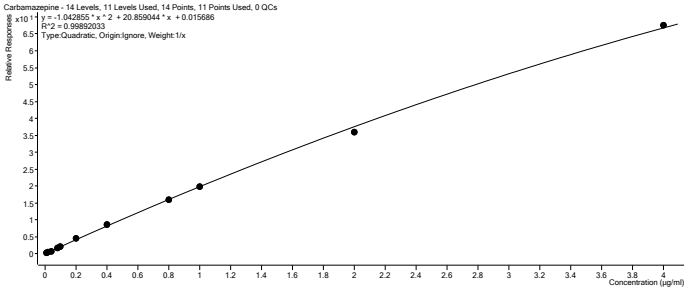


Figure 7a. Calibration curve for carbamazepine in dried blood spots.

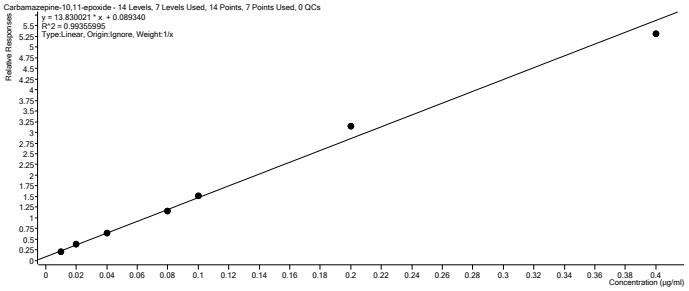


Figure 7b. Calibration curve for carbamazepine-10,11-epoxide in dried blood spots.

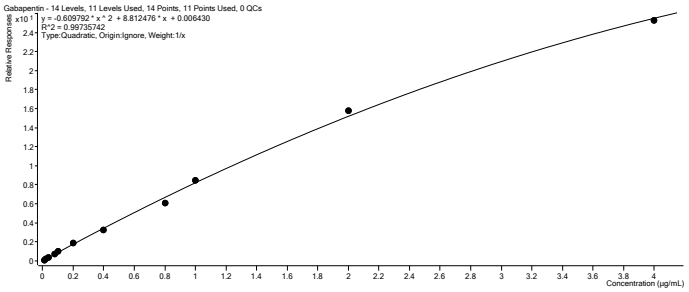


Figure 7c. Calibration curve for gabapentin in dried blood spots.

Compound	Calibration range [ng/mL]	Estimated limit of quantification [ng/mL]	Precision @ 130 ng/mL [%], n=8	Precision @ 1600 ng/mL [%], n=8	Precision real sample 1 [%], n=5	Precision real sample 2 [%], n=5	Accuracy @ 130 ng/mL [%], n=8	Accuracy @ 1600 ng/mL [%], n=8
Carbamazepine	10-4000*	8	5.2	5.2	3.1[a]	11.0[c]	94	88
Carbamazepine-10,11-epoxide	10-400	4	5.5	n.a.[+]	5.5[a]	6.8[c]	84	n.a.[+]
Gabapentin	10-4000*	2	6.6	7.0	10.9[b]	8.5[c]	112	89

Table 2. Method performance data.
** quadratic calibration function*
[+] concentration outside the calibration range
[a] multiple sampling with HemaXis DB 5 h after intake
[b] multiple sampling with HemaXis DB 6 h after intake
[c] multiple sampling with HemaXis DB 28 h after intake

Figures 8a to c show the concentration time curves for the study participant's capillary blood for carbamazepine, its metabolite carbamazepine-10,11-epoxide and gabapentin after intake of a single dose of a 200 mg carbamazepine retard or a 300 mg gabapentin tablet, respectively. The data points for carbamazepine represent the average of a double sampling, some points represent the average of a five-fold sampling. As expected the concentrations for all compounds were zero at the beginning of the study before the intake of the drug.

In the first hours the carbamazepine concentration quickly rose, reaching a maximum of around 1800 ng/mL between 13 and 33 h before gradually decreasing to around 400 ng/mL after 140 to 160 h. The concentration of the metabolite carbamazepine-10,11-epoxide increased quickly as well, reaching a maximum of around 80 ng/mL after between 33 and 48 h. The concentration maximum of the metabolite occurred later than the maximum of the parent compound. Moreover, the parent compound concentration was significantly higher than the metabolite concentration over the course of the study. Our data seem plausible when comparing with results reported in literature [12] in which average maximum plasma concentrations of around 3500 ng/mL after 25 h for carbamazepine and 250 ng/mL after 38 h for carbamazepine-10,11-epoxide are reported after a single oral dose of a 500 mg carbamazepine retard tablet.

Unlike the gabapentin tablet used, the carbamazepine tablet was a sustained-release formulation leading to a slower concentration increase in the blood samples as can be seen in the curves.

The maximum gabapentin concentration of roughly 4500 ng/mL was reached after 2.5 h. This is perfectly in-line with literature data on gabapentin pharmacokinetics reporting an average maximum concentration of around 3300 ng/mL at 2.5 h after a single intake of 300 mg of gabapentin [13].

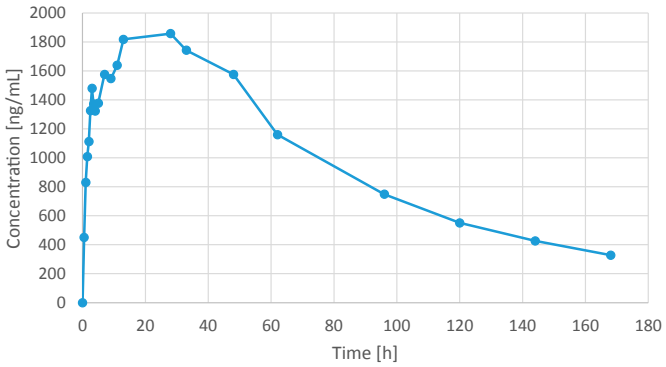


Figure 8a. Pharmacokinetic curve for carbamazepine taken by a test person as a single oral dose of a 200 mg retard tablet. The concentration values were determined in dried blood spots.

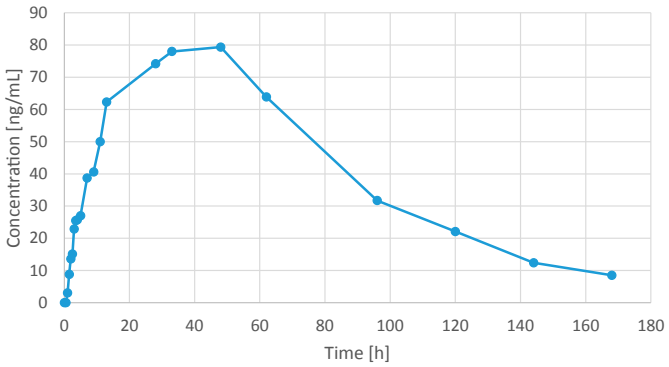


Figure 8b. Pharmacokinetic curve for carbamazepine-10,11-epoxide, a major carbamazepine metabolite, after a single oral dose of a 200 mg carbamazepine retard tablet taken by a test person. The concentration values were determined in dried blood spots.

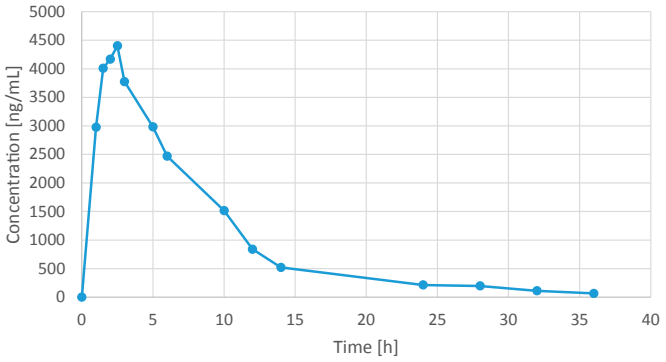


Figure 8c. Pharmacokinetic curve for gabapentin taken by a test person as a single oral dose of 300 mg. The concentration values were determined in dried blood spots.

Conclusions

In the work reported here, comprehensively automated analysis methods for the determination of carbamazepine, its metabolite carbamazepine-10,11-epoxide and gabapentin in dried blood spots were successfully developed. The methods were applied to establishing pharmacokinetic data for a single intake of the respective drugs. The data sets generated conform well to literature data showing the great potential of our methods. In addition, the methods provide good sensitivity with LOQs in the single digit ng/mL range based on samples of only 5 µL whole blood. When using the HemaXis DB10 full spot analysis is feasible with an 8 mm clamp as well. The only manual step required is putting the DBS card onto the card rack of the MPS, all further steps are fully automated. A throughput of 80 to 100 samples per day is possible with the potential to increase this number. No carry-over was observed, tubing is comprehensively rinsed between analyses. The entire analysis system was proven to be rugged and reliable.

HemaXis DB devices were employed for whole blood self-sampling enabling self-determined sampling independent of time and place, thereby making it easier to sample at multiple time points compared to conventional venous blood sampling. Relative standard deviations for sampling using HemaXis DB were between 3.1 and 11%, comparable, in our experience, to RSDs achieved using a pipette for blood spotting . The polymer envelope of the device enables safe shipping and storing of the DBS cards.

HemaXis DX devices for plasma sampling from a finger prick are currently under development. In our view, this is a valuable development since pharmacokinetic data are normally gained from plasma.

In summary GERSTEL DBS-A and HemaXis help reduce cost for sampling, shipping, laboratory handling and storage compared to conventional techniques used in pharmacokinetic studies that rely on venous blood sampling. In addition, these new techniques can help provide improved data quality since repeat or more frequent self-sampling is feasible everywhere without the need for medically trained personnel.

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