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Microassay of Drugs and Modern Measurement Techniques

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Abstract

Details are presented of the development of conventional analytical methods for the determination of drugs in paediatric plasma/serum samples via microassays. Examples of the development of small volume sampling and the use of the newer detection systems such as LC/MS/MS for enhanced detection are presented. Dried Blood Spot sampling has conventionally been used for the study of inborn errors of metabolism using Guthrie cards. Limited applications in the area of drug level determination for example in TDM had been reported but the methodology had not been widely used up until relatively. In the last few years there has been a resurgence of interest in this methodology for drug level determinations and examples are presented of drug analysis in paediatric and neonatal patients where the small volume samples are particularly useful. The application of the methodology in pharmacokinetic/ pharmacodynamic studies is discussed. The utilisation of Solid Phase Micro Extraction and Stir Bar Sorptive Extraction in drug analysis is presented. Clinical applications of these methodologies are reported including the development of in vivo Solid Phase Micro Extraction.

Introduction

Within the neonatal and paediatric population it is well known that unlicensed and off-label drug use is widespread (1-6). There have been numerous attempts to overcome these difficulties with such proposals as the FDA modernisation act of 1997 (7), the Best Pharmaceuticals for Children Act 2002 (8) and the Pediatric Research Equity Act 2003 (9). Within the European Union the EU Regulation on Paediatric Medicines (10) was adopted in 2006 and came into force in 2007. These proposals are aimed not only at providing licensing information for older, off-patent drugs but also ensuring that drugs being brought to the market are licensed for children, if appropriate.

In order for licensing data and age-appropriate dosing guidelines to be drawn up for this population it is necessary for pharmacokinetic/pharmacodynamic studies to be undertaken. In pharmacokinetic studies the drug level concentrations are determined in biological matrices and it is this aspect that will form the basis of this review, with a focus on the trend to develop micro-analytical methods suitable for studies in children and neonates.

Determination of drugs in biological matrices

In the study of pharmacokinetics/pharmacodynamics established procedures involve the determination of the drug/metabolites in plasma/serum samples. In early papers in this area it was often the case that relatively large volumes of serum/plasma were used in the analytical methodology. One paper by Roure et al (11) details a study of the pharmacokinetics of alfentanil in children undergoing surgery. The methodology describes the collection of twelve 2 ml blood samples over a five-hour period for use in the radioimmunoassay, which appears to require 1 mL of plasma although it should be pointed out that the limit of detection was 0.1 ng/mL. Similarly, in the early days of high performance liquid chromatography (HPLC), due

to the sensitivity of the detection systems and the need to determine the low [clinical] concentrations of the drug during the elimination phase in pharmacokinetic studies, relatively large volumes of biological matrices were required. For example Cooper et al (12) reported a method for the determination of hydrochlorothiazide in serum and urine. The determination utilises 1 mL of serum and involves liquid-liquid extraction, the chromatographic method involved reverse phase HPLC with UV detection (271 nm) and the method was linear in the range 200-800 ng/mL. These authors comment, even in 1976, on the volume of serum used and indicate that that in their method the volume of serum can be reduced to less than 200 µL for studies in children, thus suggesting the need for micro-analytical methods for such studies in children even at that time. It is worth noting that a recent article (13) described the determination of hydrochlorothiazide (and metoprolol) by an LC/MS/MS requiring only 100 µL of plasma with a linear of the range 3 -1000 ng/mL thus showing the changes in methodology and sensitivity over 35 years.

It is now generally accepted that in developing assays for pharmacokinetic studies in children there is a necessity to utilise sampling techniques that require as small a sample volume as possible. In order to review the developments of modern micro-analytical methods the following sections will focus on chromatographic methods since in recent history these are the mostly widely used techniques with the following aspects detailed:-

1. The analysis of drugs in plasma/serum,
2. The development of methods that have utilised Dried Blood Spots (DBS),
3. Sampling and analysis involving Solid Phase Micro Extraction (SPME) or Stir Bar Sorptive Extraction (SBSE).

Determination of drugs in in plasma/serum

Chromatographic based micro-analytical methods are widely used for the determination of drug level concentrations in plasma and serum. In such determinations nowadays Gas Chromatography (with conventional detectors [e.g. Flame Ionisation Detector]), GC/MS or GC/MS/MS, HPLC with UV/Fluorescence detection, LC/MS and LC/MS/MS as well as Ultra Performance Liquid Chromatography (UPLC) are used. Sample preparation methods have also developed over the years and although liquid-liquid extractions (LLE) are still employed other more efficient methods such as Solid Phase Extraction (SPE), Protein Precipitation (PP) and "direct" methods have come to the fore.

Indometacin is a drug used in neonates for the induction of closure of patent ductus arteriosus and because of its narrow therapeutic window monitoring is recommended. Thomas et al (14) have detailed the determination of this drug via LLE from serum or plasma followed by determination using GC/MS/MS. Either 200 µL of serum or plasma is treated with the internal standard (IS) solution, buffer and extracted with dichloromethane. The organic layer is separated and evaporated to dryness and the residue silylated. The silylated samples were then analysed by GC/MS using selected ion monitoring (SIM) with linearity over the range 0.25 – 10 mg/mL. Similarly Dailly et al (15) have reported the use of LLE in a HPLC determination of domperidone in plasma in a pharmacokinetic study involving pre-term neonates. In this method 400 µL of plasma was used in the extraction and analysis involved reverse phase HPLC and florescence detection resulting in a LOQ of 2 ng/mL although the linear range was not detailed.

Al-Asmari et al (16) have reported a method for the determination of diamorphine (DIM) and metabolites in plasma; the method being applied in a study of the use of intranasal administration of diamorphine in children with acute to severe pain. The method involved SPE with $\leq$ 250 µL plasma, which was devised so as to minimise hydrolysis of DIM during

the procedure and HPLC/MS/MS. The method used was capable of determining diamorphine and metabolites and used gradient elution and detection involving electrospray ionisation in the positive ion mode with MRM detection. The paper is worthy of study for details of the extensive validation including a study of matrix effects. Skolnik et al (17) have developed a HPLC/MS/MS method involving SPE for the simultaneous determination of both vincristine and actinomycin D [which are used in the treatment of various paediatric cancers] in paediatric plasma samples. The method utilises 500 μL of plasma which was mixed with IS standard solution and then subjected to SPE. The LC/MS/MS involved reverse phase chromatography and detection using ESI (positive ion mode) and MRM. The method is reported to be linear, for both compounds over the range 0.5 – 100ng/mL with 0.5ng/mL being the LLOQ. Similarly Herd et al (18) have reported a LC/MS/MS method for the determination of ketamine and norketamine in children utilising SPE. In this methodology a 200 μL blood sample was mixed with 50 μL of IS solution containing deuterated ketamine and norketamine and 3 mL of phosphate buffer. The solution was sonicated for 15 min, centrifuged and the subjected to SPE. The reverse phase HPLC incorporated a MS system comprising a Turbolon spray source used in the positive ion mode with MRM. The method was linear for ketamine over the range 0.125 – 4.5 mg/mL and 0.06 – 2.0 mg/mL for norketamine.

Salm et al (19) have detailed a LC/MS/MS for the determination of tacrolimus and cyclosporin in whole blood. These workers comment on the problems associated with conventional immunoassay for these two compounds due to cross reactivity with metabolites and difficulties with low hematocrit on the determination of tacrolimus. PP was carried out by the addition of a zinc sulphate solution to a 100 μL blood sample, followed by the addition of a solution containing two IS (ascomycin and cyclosporine D), this mixture was simply mixed, centrifuged and the supernatant injected onto the LC/MS/MS system. The reverse phase

HPLC incorporated APCI used in the negative ionisation mode. The method was linear for tacrolimus over the range 1–30 μg/L and 2–2000 μg/L for cyclosporin.

Muller et al (20) have investigated the pharmacokinetics of penicillin G in infants with a gestational age of less than 32 weeks using a simple HPLC method with UV detection (215 nm). The method utilises only 100 μL of serum and the sample prep is straightforward involving addition of IS solution (methicillin in methanol), vortexing for 30 s, cooling at -20 ^{o}C for 10 min, vortexing again for 30 s, centrifugation and filtration before injection of the supernatant. Similarly Metsvaht et al (21) have described a pharmacokinetic study of penicillin G in very low birth weight neonates. The analytical method is similar to that detailed above [HPLC/UV detection at 200 nm] but uses only 50 μl of plasma, which is treated with acetonitrile, vortexed and centrifuged and then the supernatant is injected onto the HPLC system.

In GC it is often the case that derivatisation techniques are necessary (e.g. in the indometacin assay above) in order to produce satisfactory chromatography, however, in HPLC derivatisation is rarely required to optimise the chromatography because of the larger number of parameters that can be modified in HPLC compared to GC. In some cases, however, researchers have reported the HPLC analysis of drugs using derivatisation methods. Topiramate (Figure 1), an anticonvulsant drug, has no chromophore and thus is inactive in terms of UV/visible or fluorescence detection. Vovk et al (22) have detailed a method for the determination of topiramate in plasma that is applicable to pharmacokinetic studies in both adults and children with epilepsy. This method involves derivatisation using 9-fluorenylmethyl chloroformate (Fig 1; 2), which reacts with the amide function of topiramate to give a fluorescent label so that the compound can be determined with high sensitivity. The method (based on the work of Bahrami et al (23)) involves a simple extraction of topiramate from 250 μL of plasma with dichloromethane, the organic extract was evaporated to dryness

and the residue treated with 9-fluorenylmethyl chloroformate in the presence of borate buffer. The reverse phase HPLC methodology employed spectroflurometric detection and the method was linear over the range 0.1–15 mg/L.

The details presented above have shown the development of micro-analytical methods utilising small volumes of biological matrices and innovative extraction methods. Despite these developments paediatric researchers have looked to develop newer methods where even lower sample volumes are employed and novel sample methods from other analytical areas have been investigated.

Dried Blood Spot sampling

History

Dr Robert Guthrie was an American scientist who had a particular interest in the genetic disorder phenylketonuria (PKU). With PKU there is a lack of the enzyme phenylalanine hydrolase, which results in a build up of phenylalanine in the body and this can lead to a number of serious problems including mental retardation. Guthrie (24) was searching for a simple method of blood sampling in neonates so that the levels of phenylalanine could be determined to check for PKU and therefore dealt with to prevent the known problems. This led to the development of the "Guthrie Card" as detailed in Figure 2.

Use for drug assay

Nowadays these cards are used to collect heel prick blood samples from neonates during their first few days of life, the samples are then sent to a central laboratory where they can be screened for a whole series of inborn errors of metabolism including PKU, congenital hypothyroidism, cystic fibrosis and sickle cell disorders. Following their use in the investigation of inborn errors of metabolism other workers sought to utilise this sample collection method to determine drug concentrations in whole blood samples. The first

example reported in 1978 was the use of DBS for the determination of theophylline (25). Later an alternative EMIT method for the determination of theophylline in DBS (26) was reported and this was applied to the study theophylline levels in asthmatic children and the acceptance of domicilary DBS collection (27). During the 1980/90s further, intermittent applications were reported detailing the determinations of for example chloroquine (28), mefloquine (29) sisomicin (30), gentamicin and netilmicin (31), quinine, hydroxychloroquine, chloroquine, and desethylchloroquine (32), dapsone, monoacetyldapsone, and pyrimethamine (33).

Over the last ten years applications specifically related to the use of DBS sampling in clinical areas, therapeutic drug monitoring (TDM) and more specifically paediatric studies began to appear (34-36). In one of these articles Oliveira et al (35) reported the determination of paracetamol and its main metabolites in dried blood spots, the developed method being applied to the analysis of DBS samples obtained from neonates. The method involved reverse phase HPLC with UV detection and was capable of determining paracetamol (PA), paracetamol glucuronide (PAG) and paracetamol sulphate (PAS). The DBS were extracted using a formate buffer solution, the IS was then added followed by acetonitrile for protein precipitation, this solution was then centrifuged and the supernatant evaporated to dryness before reconstitution in formate buffer. The analytical range for the 3 compounds was reported to be 40–2000 ng/mL for PA and 160–4000 ng/mL for PAG and PAS. The authors report the application of the methodology in neonates by presenting the PA concentration time profile for a neonate following a rectal dose of PA.

Damen et al (37) have recently reported an updated HPLC method for the determination of vincristine and actinomycin-D in dried blood spots. In this method the preparation of standard DBS samples involved the spiking of whole blood (collected from healthy volunteers) and the application of 40 µL volumes onto DBS cards. From these samples circles 0.25 inches in diameter were cut, extracted with acetonitrile/methanol/water (1:1:1, v/v/v) containing IS for

15 min with sonification. Following the extraction the sample was simply centrifuged and the clear supernatant injected onto the LC/MS/MS, which employed electrospray ionisation detection operating in the positive ion mode and MRM. The assay for vincristine was linear from 1 to 100 ng/mL and actinomycin-D from 2 to 250 ng/mL and was tested on samples obtained by a finger prick and the authors indicating the applicability of the method to routine clinical investigations. Although this methodology was not specifically developed for paediatric studies it is worth comparing the method with that above for a similar determination of these same two drugs in plasma by Skolnik et al (17). In the plasma method 500 mL was used which would mean that 1 mL of blood was needed whereas in the DBS method only 40 µL was employed and in fact only a portion [probably 10-12 µL based on an approximate calculation] of this was used for the assay. Despite the much smaller sample volume used in the DBS methodology the linear range is virtually the same.

Lindkvist et al (38) have recently reported the utilisation of DBS sampling for the determination of sulfadoxine and sulfamethoxazole in children for a compliance study. The authors indicate that with malaria treatment failure may be due to drug resistance or poor compliance and thus have developed a simple DBS based sampling method to study these aspects. The DBS extraction process was a simple aqueous extraction using dilute perchloric acid, shaking for 30 min and centrifugation; this was followed by HPLC analysis incorporating UV detection. The authors indicate that the developed method was applicable for compliance studies with these two drugs.

There are numerous reports of the use of DBS sampling in HIV studies in children both for the diagnosis of HIV infection and also for TDM of antiretroviral drugs. One of the tests used for testing babies born to HIV infected mothers involves a Polymerase Chain Reaction method which tests for HIV DNA this can now be accomplished using DBS (39). Amongst the plethora of papers detailing the determination of antiretroviral drugs is the recent paper by Meesters et al (40), this describes a matrix-assisted laser desorption/ionization-triple

quadrupole tandem mass spectrometry (MALDI-QqQ-MS/MS) for the determination of lopinavir and ritonavir concentrations in DBS samples. These authors indicate that the time for a single determination (once extracted) is only 15 seconds; which means that a whole 96 well sample tray can be analysed in approximately 17 minutes.

Within the last two years a number of articles have appeared discussing the use of dried blood in pharmacokinetic studies with several specifically focusing on children and neonates (41-45). Spooner et al (41) have detailed the development of a DBS method for paracetamol, which they describe as being appropriate for pharmacokinetic studies, the methodology involves simple extraction followed by HPLC/MS/MS using SRM. The linear range was 25-5000 ng/mL and the technique was applied to a clinical study involving human volunteers. Patel et al discuss the various aspects of the use of DBS sampling in order to facilitate pharmacokinetic studies in children (42) and describe a LC-MS method for the determination of dexamethasone in DBS (43), which is reported to be particularly suited to pharmacokinetic studies involving paediatric populations. Suyagh at have also described the application of DBS sampling methods for the determination of canrenone (44) levels in a pediatric population and metronidazole (45) in neonates, both methods being specifically developed for pharmacokinetic studies. The latter methodology has been utilised in what is believed to be the first published pharmacokinetic study employing DBS (46).

Dried blood spot concerns

Although there have been many recent developments in DBS sampling for drug level determinations in both the adult and the paediatric/neonatal population there are a various aspects that need consideration if this technique is to become a widely applied technique in TDM and pharmacokinetic/pharmacodynamic investigations. Firstly the technique does appear to be most valuable in relation to the micro-analytical aspects, which are the focus of this review, in that many of the recent papers detail a sample volume of 30 μL or less. Despite this low volume reports indicate that the methodology is more than sensitive for the

TDM/clinical investigations, LC/MS methods seem to be the norm with the added sensitivity this brings although Suyagh et al (45), along with others have effectively utilized HPLC with UV/Fluorescence detection. There is ample evidence to suggest that there has been extensive investigation of the validation of the analytical methods in terms FDA/ICH requirements (41,43-45), although the validations seem to have followed the general "bioanalytical" standards and not any that are specific to DBS analysis. A number of papers have detailed aspects that need to be looked into in relation to successful DBS applications in particular the sampling paper used [and whether this is pre-treated or not], the effect of different volumes of dried blood being applied to the sampling paper, the preparation of standard DBS samples in relation the patient samples, the effect of haematocrit on the sample application, the extraction of the drug from the sampling paper and the stability of samples once dried on the sample paper (41,47-50). At present it is, in the opinion of the author, impossible to specify a standard approach to the development of DBS analytical procedures for TDM and clinical studies although work to rectify is being conducted in Queens's University Belfast.

Dried blood spot use in PK studies

A second aspect of the DBS methodology is the utilisation of the data once obtained especially in the case of pharmacokinetic studies. The data generated from the DBS analysis will give whole blood concentrations whereas the majority of pharmacokinetic data will have previously been based on plasma/serum data. Various authors have developed DBS methodology and compared the results with plasma or serum values. In some instances it has been reported that the relationship between the concentrations in the two biological matrices is well correlated and of similar levels for tacrolimus and lamotrigine respectively (51-53) whereas in other instances although the results are correlated with the determined levels are somewhat different (54-5) and in need of correction e.g. homocycteine (54), serotonin, 5-hydroxy indolacetic acid and homovanillic acid (55) and phenobarbital (56). This variation in DBS/plasma[serum] concentrations has been suggested to be based on the blood/plasma ratio of the drugs and the hematocrit. The implementation of corrections is discussed in some detail

by Li and Tse (48) who also indicate that the fraction of drug bound to blood should be taken into account although they do indicate possible problems in this area. Emmons and Rowland (57) have also produced an interesting commentary on the use of DBS data in place plasma/serum data for pharmacokinetic studies and suggest some possible approaches to overcoming these difficulties. It is also apparent that even with these approaches there are other important aspects that will also need addressing before the DBS sampling can be routinely used in clinical/pharmacokinetic in place of plasma/serum sampling.

Solid Phase Micro Extraction and Stir Bar Sorptive Extraction

Solid Phase Micro Extraction (SPME) and Stir Bar Sorptive Extraction (SBSE) are sample collection and clean up procedures that have been developed for a range of different areas but which are now finding applications in drug analysis. These techniques have been applied in many areas for studying environmental samples, volatile organic compounds, food and wine sampling etc but it is only in the relatively recent past that their application in drug analysis is being fully realised.

Solid Phase Micro Extraction

SPME was developed by Arthur and Pawliszyn (61) and is a sample preparation technique based on the adsorption of analytes directly from an aqueous sample onto a coated, fused-silica fiber. This sampling technique is fast, easy to use and eliminates the use of organic solvents. The SPME fiber can be inserted into the liquid sample or suspended above the sample for headspace sampling. The fused silica fiber is coated with an appropriate coating for the extraction and in much of the early work polydimethylsiloxane (PDMS) was used; this was appropriate since many of the compounds under investigation were highly lipophilic. Once the compound(s) have been extracted onto the coated fiber they can then be analysed by GC or HPLC, in the simplest examples the coated fiber in introduced into the heated injection port of a GC and the fiber is exposed and thermal desorption of the sample occurs,

alternatively the fiber can be introduced into a small volume solvent chamber for desorption into solvent which can then be injected into a HPLC. There are a variety of factors that can be investigated in order to optimise the extraction e.g. temperature, time, desorption conditions etc., however, once optimised the extractions can be shown to produce excellent results. Several excellent reports on the details of the basic theory and method optimization in SPME in relation to bioanalytical methodology have been published (62-67)

Nagasawa et al (68) reported the use of PDMS fibers for the extraction of nicotine, cotinine, amphetamine, methamphetamine in blood using head space SPME and GC/MS. The blood sample in a sealed vial was heated at 80 °C for 20 min and the SPME fiber was exposed for 5 min in the headspace of the vial. Following extraction the fiber was introduced into the injection port of the GC-MS where the analytes were firstly derivatised to form heptafluoro-butyramide (HFB) derivatives, which were then determined, using SIM. As indicated initial studies in the area of SPME were based on PDMS coated fibers that are well suited to the extraction of highly lipophilic compounds, however, many drugs are polar and thus there has been a need to develop alternative coating materials for extraction. Unceta et al (69) have reported the simultaneous determination of citalopram, fluoxetine and metabolites in human urine samples by solid-phase microextraction using HPLC. In this paper (typical of many such papers) the authors investigated the optimisation of the conditions of extraction, the factors of importance that they considered were type of fiber, speed of stirring (fixed but unstated), temperature (extraction was carried out at 21^{o}C), time of extraction, ionic strength and pH, organic modifier (and percentage) and desorption process. The first factor investigated was the type of fiber to be used and three were studied namely Polyacrylate, PDMS/DVB and Carbowax/Templated Resin and over the whole range of compounds the Carbowax/Templated Resin was the most appropriate. The paper describes other optimisation factors and is well worth study for an insight into this aspect for prospective users of this methodology. The analytical method developed proved most satisfactory for the determination of the drugs in urine samples.

A range of drugs have been analysed using SPME including ibuprofen (70) and ketamine (71) in urine, antidepressants in plasma (72,73) [with the second example here demonstrating the use of in-tube SPME] and antibiotics in whole blood and plasma (74). Interestingly there have been a number of applications where SPME has been employed to determine the levels of propofol (75, 76) and fentanyl (77) in the exhaled breath of anaesthetised patients. In the above examples there has been an indication of the use of small volume sampling in SPME but not such low volumes as have been applied in the conventional and DBS sampling discussed above, additionally there are few if any specific applications in the pediatric area. The application of this methodology to the determination of drugs in biological matrices is continuing and it is likely that it will find application in the pediatric and neonatal in the near future. One further area under study at the present is the development of SPME fibers, along with accompanying protocols, for the determination of drugs *in vivo*. There are several excellent papers (78-80) in this area, which discuss the problems associated with the development of appropriate biocompatible fibers and the procedures for the validation of the analysis and the application in for example pharmacokinetic studies. To the authors best knowledge these methodologies have been applied in animal studies but as yet no human applications have been reported.

Stir Bar Sorptive Extraction

Stir-bar sorptive extraction is a solvent-less sample preparation method for the extraction and enrichment of organic compounds from aqueous matrices. The method is based on the same principles as solid-phase SPME and was developed by Sandra and colleagues in 1999 (81). Compared with SPME, a relatively large amount of extracting phase (PDMS) is coated on a stir bar. Solutes are extracted into the coating, based upon their [octanol/water] partitioning coefficient and upon the sample extraction medium phase ratio. The technique has been applied successfully to trace analysis in environmental, biomedical and food applications and extremely low detection limits have been reported. Most recently, SBSE has been used for extraction of drugs from various biological matrices such as urine (82-83), plasma (84-86)

and urine, plasma and brain tissues (87). Chaves et al (88) have described the determination of a range of antidepressants in plasma and hve detailed a set of procedures for the optimisation of the analysis, again researchers wishing to look at method development in this area could usefully check on this reference. Again in these examples there are little or no paediatric examples and once again the samples volumes utilised in the analyses are relatively large, however, once again it may well be possible to utilise smaller sample volumes as the methodology improves and indeed most recently a method has been developed for the determination of diclofenac in paediatric urine samples using this SBSE approach (89).

Conclusions

Thus it is clear that micro-analytical methods have become very important for paediatric/neonatal clinical/pharmacokinetic/toxicology studies. From the evidence presented above it is clear that conventional studies involving plasma/serum sampling have benefited from some of the developments in both sample preparation (SPE etc) and instrumental (MS and MS/MS) detection systems such that lower sample volumes (50-100 μL) are becoming common. The advent of DBS sampling has meant that even lower sample volumes (15-20 μL) are useable. There is also evidence that novel and automated methods are being developed for this work (58-60). These advances in the application of DBS sampling will see the utilisation of this methodology become more common in the future although the interpretation of DBS concentrations versus plasma data will need substantial studies to investigate the factors raised by Li and Tse (48) and Emmons and Rowlands (57). SPME and SBSE have been shown to be useful in drug analysis and it is likely that these extraction methodologies will become widespread in paediatric analysis in the near future. The development of *in vivo* sampling techniques utilising SPME will most likely be a very important tool especially in pharmacokinetic analysis, however, the protocols will again need to developed so that comparison with presently available plasma/serum data can thoroughly

investigated (78-80).

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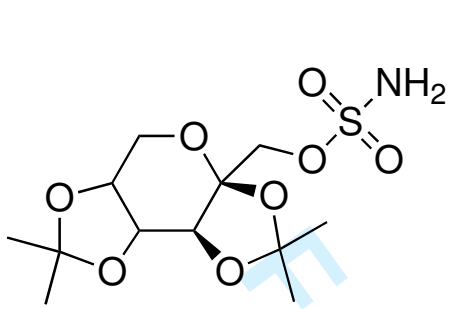
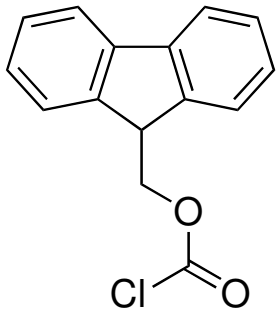
Table 1. List of abbreviations

Terminology	*Abbreviation*
High Performance Liquid Chromatography	HPLC
Ultra Performance Liquid Chromatography	UPLC
Liquid Chromatography/Mass Spectrometry/ Mass Spectrometry	LC/MS/MS
Dried Blood Spot	DBS
Gas Chromatography/Mass Spectrometry/ Mass Spectrometry	GC/MS/MS
Liquid Liquid Extraction	LLE
Protein Precipitation	PP
Solid Phase Extraction	SPE
Limit of Quantification	LOQ
Multiple Reaction Monitoring	MRM
Internal Standard	IS
Atmospheric Pressure Chemical Ionisation	APCI
Gas Chromatography	GC
Enzyme-Multiplied Immunoassay Technique	EMIT
Selected Reaction Monitoring	SRM
Solid Phase Microextraction	SPME
Stir Bar Sorptive Extraction	SBSE

Figure 1. Structures of Topiramate [1] and 9-fluorenylmethyl chloroformate [2]



1



2

Figure 2. Guthrie card used in DBS Sampling