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Review

Therapeutic drug monitoring and LC–MS/MS☆

Joanne E. Adaway[a,b,*], Brian G. Keevil[a,b]

[a] Department of Clinical Biochemistry, University Hospital South Manchester NHS Foundation Trust, Wythenshawe Hospital, Southmoor Road, Wythenshawe, Manchester M23 9LT, UK
[b] University of Manchester, Manchester Academic Health Science Centre, Wythenshawe Hospital, Southmoor Road, Wythenshawe, Manchester M23 9LT, UK

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ABSTRACT

LC–MS/MS is an increasingly important tool in therapeutic drug monitoring as it offers increased sensitivity and specificity compared to other methods, and may be the only viable method for quantifying drugs without natural chromophores or fluorophores. The choice of sample preparation method, column technology, internal standard and mass spectrometric conditions is important to ensure accurate drug measurement and to avoid interference from matrix effects and drug metabolites. LC–MS/MS is a more involved technique than automated immunoassays, but technological advances such as the development of pipetting robots and online solid phase extraction mean that LC–MS/MS is becoming an attractive and convenient method for therapeutic drug monitoring in clinical laboratories.

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1. Introduction

Therapeutic drug monitoring (TDM) is required to optimise therapy of critical dose drugs with a narrow therapeutic range where there is a good chance of either overdosage or underdosage. Monitoring the drug concentration can guide the drug dosage to optimise therapeutic effectiveness whilst minimising the side effects. TDM has been performed for many years using immunoassay but it is recognised that immunoassay methods can suffer with non-specific interference from related compounds, metabolite interference or matrix effects.

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) has been in routine use in clinical laboratories for a little over 10 years, neonatal screening laboratories apart, and is becoming an increasingly important technique for the analysis for the measurement of prescribed drugs. LC–MS/MS offers improved specificity and sensitivity and it offers the only viable measurement technique besides immunoassay for compounds without natural chromophores or fluorophores. Immunoassay has many problems [1], and LC–MS/MS is regarded as a superior

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* Corresponding author at: Department of Clinical Biochemistry, University Hospital South Manchester NHS Foundation Trust, Wythenshawe Hospital, Southmoor Road, Wythenshawe, Manchester M23 9LT, UK.
E-mail addresses: Jo.adaway@uhsm.nhs.uk (J.E. Adaway), Brian.keevil@uhsm.nhs.uk (B.G. Keevil).

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technique to immunoassay because of its better specificity and sensitivity, but the main disadvantages of LC–MS/MS are seen to be the high instrument costs, greater technical complexity, speed and turnaround of analysis. For these reasons demand for LC–MS/MS has been strongest when used to measure analytes where there has been no suitable analytical alternative, e.g. routine monitoring of immunosuppressive drugs by LC–MS/MS was instigated because immunoassay methods suffered from metabolite interference. The situation for measurement of some other drug classes is different because immunoassay methods may not be available or UV based HPLC methods may lack sensitivity, e.g. HIV drugs. In respect to TDM, LC–MS/MS is not available in all centres but is increasingly being used for specialist analysis in referral centres for infrequently requested or difficult to measure drugs [2].

1.1. Specimen collection

The commonest sample type used routinely for TDM is either plasma or serum. Hydrophobic drugs such as ciclosporin partition into red cells and the recommended sample is venous whole blood, although finger prick capillary samples have been shown to be a good alternative. There have also been some reported methods using other body compartments such as saliva and peripheral blood mononuclear cells although these alternative sampling strategies have not gained widespread use. Correct sample handling of blood can be important to minimise degradation of drugs or drug metabolites, for example samples for MPA measurement must be centrifuged and the plasma separated from red cells soon after sampling to avoid degradation of the drug.

1.2. Sample preparation

There is a general requirement to remove at least protein and preferably other interfering substances from the sample before analysis. Protein can cause problems with blockages of frits and injectors although some systems are designed to overcome this. It is wise to eliminate matrix effects as much as possible because these can have deleterious effects on the analysis [3,4]. Matrix effects are the alteration of ionisation efficiency caused by co-eluting substances, mainly salts and phospholipids. Salts are relatively easy to remove whereas phospholipids are difficult to remove effectively even with sophisticated sample clean up procedures. The most popular sample treatment strategies comprise solid-phase extraction (SPE), liquid–liquid extraction (LLE) and protein precipitation (PP). Strategies to identify and eliminate matrix effects in LC–MS/MS assays for drugs have recently been reviewed by van Eekhaut et al. [5]. The choice of clean up technique relies on chemical characteristics of the analyte and achieving good recoveries of drugs is largely dependent on the polarity of the drug. LLE is carried out on plasma samples with immiscible organic solvents such as diethyl ether, ethyl acetate, methyl tert-butyl ether, and hexane. High recoveries and clean extracts with negligible matrix effects can be obtained depending on the solvent chosen. LLE is probably the most effective way of removing matrix effects because ionised compounds, e.g. some phospholipids do not partition readily into the organic phase. This is also true for ionised drugs and metabolites and in these cases SPE or PP may be a better alternative. LLE is very efficient but is difficult to automate, can be time consuming and there is also a need for fume extraction to remove solvent vapour, which is not always available in routine laboratories. SPE relies on trapping the analyte on immobilised media and then washing proteins and matrix interference to waste, the analyte is then eluted with an organic solvent, usually methanol or acetonitrile. The SPE devices available now cover a wide range of chemistries including hydrophobic, ion exchange and mixed mode stationary phases and if care is taken to match the analyte to the appropriate stationary phase then clean extracts can be obtained. SPE has wider applicability than LLE because it can also adsorb ionic compounds but the main drawback is the cost of the columns. Columns can be supplied as individual units for manual use and also in 96-well plate format for use with robotic sample processors. This is referred to as off line SPE whereby the samples are prepared away from the LC–MS/MS instrument. SPE can also be used in an on line manner whereby the analyte is trapped using a small column, washed with weak solvent and then eluted with strong solvent after column switching onto an analytical column. PP is usually needed prior to this to prevent instrument blockages but there are commercially available systems that perform a similar task, without the need for PP. One of these systems uses disposable extraction cartridges (Symbiosis, Spark Holland, Netherlands) and the other uses turbulent flow technology (Aria, ThermoFisher Scientific, USA). PP is the simplest technique to use; it can be easily automated and can be performed in 96-well plates to prevent needless sample transfer steps. The main disadvantages are a relatively dirtier sample extract which is also diluted by the solvents used for precipitation, and this may lead to assay sensitivity problems. There are now commercially available protein precipitation devices in plate format that allow PP within the plate whilst also removing phospholipid. Examples of such protein precipitation plates include Waters Ostro™ (Waters Corporation, USA) and HybridSPE™ (Supelco Analytical, Sigma Aldrich, USA). These devices also allow collection of the protein free analyte in a collecting plate for direct injection or concentration, without the need for centrifugation. The main advantage of SPE and LLE over PP, apart from providing a cleaner extract, is that they can both provide a sample concentration step if assay sensitivity is an issue. The need to turn the results around in a timely fashion is also an important consideration when deciding on the type and extent of sample clean up required. It may well be the case that PP for a particular drug analysis gives a clean enough extract with sufficient sensitivity, but careful validation of the method needs to carried out to confirm this.

1.3. LC columns

Good chromatography is essential for the development of robust methods. The validation process should include optimisation of analyte, retention on the column and the demonstration of clean chromatograms with no isobaric interference. Matrix effects should be thoroughly investigated with infusion experiments using the analyte in question to test qualitatively for ion suppression. A more quantitative approach to assess for matrix effects can be achieved by comparing the recovery of spiked analyte from aqueous and plasma samples [5]. It is also possible to monitor interferences directly using targeted analysis of individual phospholipids [6] either in a qualitative or quantitative manner. This approach can also be very effective when trying to determine the most appropriate chromatographic conditions for removing ion suppression. A variety of column chemistries are now available and these also come in a variety of particle sizes from 5 μm down to 3 μm for analytical work. It has been a constant problem with the smaller particle size columns that better resolution was achieved with higher back pressure and this could be a limiting factor in the choice of column because of leakages. There is now a trend to use sub-2 μm particles to increase peak resolution and sensitivity and this has been made possible with an improvement in LC technology to cope with the much higher back pressures generated by these systems. The fittings in these UPLC systems can withstand 5–6 times the back pressure of conventional LC systems. For those laboratories without access to UPLC equipment the use of fused core particle technology (halo) columns may provide nearly as much chromatographic separation.

There is now a huge range of column chemistries, particle sizes and column sizes to choose from. The choice of column can seem daunting but can be guided from previously published work. Often trial and error is the best way to choose but this can be an expensive option. The choice of column has a profound effect on assay performance and C18 columns from different manufacturers can be affected differently by matrix effects and hence give different results with clinical samples. Whatever the decision the method should be fully validated and the column supplier should not be changed without full validation of the new column. Columns may seem expensive to buy but with adequate sample clean-up it is not uncommon to get 2–3000 injections from a modern LC column.

1.4. Mobile phase

Solvents and additives used to make the mobile phase must be compatible with mass spectrometry. Only buffers that are volatile can be used, e.g. ammonium acetate, ammonium formate and pH modifiers, e.g. formic acid, trifluoroacetic acid or ammonia. Ion pairing reagents can be used to retain highly polar analytes but these must also be volatile, e.g. TFA, heptafluorobutyric acid may also significantly reduce the assay sensitivity by suppressing ionization [7]. Altering the mobile phase composition or pH is a useful tool, particularly for drug analysis when the pK_a of different drugs can vary widely and can have a significant effect on analyte retention.

1.5. Mass spectrometry

The most common detectors in use for TDM are triple quadrupole instruments. There are a number of vendors that supply excellent instruments but it should be noted that each vendor will have several different models ranging from entry level to high grade research instruments. The differences in instrument sensitivity and performance are wide both within and between vendors. Part of the reason for this is the physical design of the ionisation source and the temperatures at which the source operates. It is therefore difficult to make general comparisons of instrument performance because it is usually analyte specific. In general terms instrument performance has improved significantly over the past 10 years and there have been gains of up to 10-fold in instrument sensitivity. The most common ionization techniques are electrospray ionization (ESI) and atmospheric chemical ionization (APCI). ESI is an efficient method for converting analyte in solution into gas phase ions suitable for analysis by the processes of desolvation and ion desorption. This is especially good for polar analytes and is the most popular source used for drug analysis. APCI uses a much hotter ionization source with a corona discharge region where gas phase chemical reactions take place. APCI gives a more selective ionization and importantly for some compounds it has been shown to have much lower matrix effects. The triple quadrupole instrument consists of two mass analysis quadruples separated by a collision cell. The strengths of this instrument lie in the ability to filter ions of a pre-determined mass and then fragment these ions in a compound specific way. Monitoring these fragment ions which can only have come from the parent ion gives rise to high analytical specificity, but it should be remembered that when operating at near maximum resolution the instrument will only separate ions with one Dalton resolution. Isobaric interference from structurally related compounds or metabolites is therefore still possible and this can only be reduced by performing good sample clean-up and chromatography as discussed in Section 1.3. The transition chosen for analysis is determined by careful tuning and both positive and negative ion mode ionization should be explored. The instrument is tuned using pure solutions of analyte and the daughter ions are selected on the basis of abundance to give the most sensitive assay. It is now common practice to select quantifier and qualifier ions to compensate for any possible interference in the assay and to improve specificity.

The scan speeds of modern instruments are much faster and allow the acquisition of at least 15 data points across the chromatography peak for each transition, even for very narrow peaks produced by UPLC instruments. This number of points is necessary for optimal peak integration but it could be a struggle to achieve this acquisition rate with earlier instruments when multiple analytes with multiple transitions were being monitored.

Internal standards are necessary to control for fluctuating recovery caused by variable ionization within the source or the precision of the extraction procedure. A variety of internal standards has been used including structural analogues of the analyte or deuterated compounds. Analogue internal standards are often considered inferior to deuterated compounds but were often the only cost effective and practical alternative [8]. There are exceptions to this rule and the successful use of ascomycin in the assay of tacrolimus is a good example. Unless an appropriate internal standard is chosen then it is unlikely that the method will ever be robust. When clinical samples are to be measured the assay should also be validated using samples taken using blood collection tubes from other manufacturers and using different anticoagulants. These could all have an effect on how well the internal standard performs because of differing ion suppression profiles. When using a deuterated internal standard it is generally better to choose one with more than 2 substituted deuteriums. When analysing large amounts of analyte the $m+2$ peak can interfere with bi-deuterated internal standards causing an apparent reduction in calculated analyte [9]. Care must also be taken to choose an internal standard that is not interfered with by metabolites or in-source generated metabolite fragments.

Documentation detailing the specific steps for development and validation of quantitative LC–MS/MS assays has been reviewed by Honour [10].

2. Antifungal drugs

Azole antifungal agents are used for the prophylaxis and treatment of fungal infections in immunocompromised patients. The identification of concentrations that are both effective and non-toxic has been facilitated by an increased understanding of the pharmacokinetics and pharmacodynamics of azole compounds. Their use in clinical practice has been reviewed by Denning et al. [11]. The need for TDM of these drugs has been highlighted by pharmacokinetic variability, drug–drug interactions and serum concentration related toxicity.

LC–MS/MS assays have been developed for voriconazole [12,13], itraconazole [14], fluconazole [15], posaconazole [16–18] and iodiconazole [19]. Other authors have focussed on multiplexed assays to include all of the azole compounds including voriconazole, itraconazole, fluconazole and posaconazole [20–23]. There has also been a recently published method for the measurement of azoles combined with echocandins including anidulafungin, caspofungin, isavuconazole and micafungin [24]. To meet the goal of rapid analysis prevalent in clinical laboratories, many of these methods have adopted a simple protein crash followed by direct injection onto a C18 column [12,15,18,21,23–25], whereas others have used off line SPE [14,17], on line SPE [13] and liquid–liquid extraction using hexane [19]. The time of analysis varied considerably amongst the methods with many achieving run times of 2–3 min with the longest run time being the online SPE method at 13 min. A variety of internal standards have been used including structural analogues of the azoles, the azoles themselves and deuterated internal standards. Two structural analogues have been used for posaconazole, SCH 56984 [16] and 1-(1H-1,2,4-triazole)-2-(2,4-diflurophenyl)-3-[N-methyl-N-(3-chlor-benzyl)amino]-2-propanol) [19]. Ketoconazole

was used for posaconazole, itraconazole and voriconazole [20] and ketoconazole alone [12]. Itraconazole was used for posaconazole, voriconazole and isovuconazole [24]. Fluconazole [14] has been used to measure itraconazole and quinoxaline was used to quantify itraconazole, voriconazole, and posaconazole [22]. If another azole is used as an internal standard it is important to use one that is not likely to be co-prescribed. Deuterated internal standards are now more widely available and are the preferred choice; these have been used to quantify fluconazole, itraconazole, hydroxyitraconazole, posaconazole, voriconazole [21] and also fluconazole [15]. The azoles differ in hydrophobicity and there is generally good chromatographic separation of these compounds on C18 columns. Recently UPLC [21] and fused core silica particle technology columns [17] have been used to improve chromatography still further. Because of the good chromatographic separation there is a risk that there will not be sufficient compensation for ion suppression effects if only one internal standard is used for multiple analytes. Thorough assay validation is therefore necessary to investigate any matrix effects and particular attention should be paid to possible metabolite interference, e.g. posaconazole metabolites have been found to interfere in the LC–MS/MS assay but superior separation using UPLC resolved this problem [26]. In the LC–MS/MS procedure, comparably wide and left side shifted peaks were noticed and this was thought to be caused by in-source fragmentation of posaconazole glucuronides during electrospray ionisation. Reducing the cone voltage led to disappearance of the glucuronide peaks and modification of the LC–MS/MS method enabled separation of the main interference. Analysis of azoles has been performed in positive ion mode using either electrospray or APCI. All of the methods showed good sensitivity and typically required less than 100 μL of serum for analysis even when using entry level instruments. The methods using SPE have tended to use C18 based phases whereas Cunliffe [17] thought these were unsuitable and used mixed mode cation exchange. They loaded sample on to the SPE columns with acidic conditions and eluted analyte with basic conditions.

Echinocandins are larger and structurally different molecules to the azoles. There are currently three commercially available echinocandin antifungal drugs, i.e. anidulafungin, caspofungin, and micafungin. All three drugs have low oral bioavailability, high protein binding, and relatively low urinary excretion of the parent drug. The need for TDM of these drugs is still uncertain because the relation between echinocandin blood levels and treatment outcome is currently undefined. Combined assays for both azole and echocandin drugs have been described [21,24] (Table 1).

3. Anti viral drugs

TDM of HIV drugs has been recently reviewed by Checa et al. [27] and also Taylor et al. [28]. The drugs are designed to disrupt viral replication at various stages of the cycle and have been classified into 3 distinct groups which include nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs), but these are also joined by two different classes, entry inhibitors and integrase strand inhibitors. The main thrust of TDM applications has been with the NNRTI and PI drugs because the NTRIs are actually prodrugs and need phosphorylation to make them metabolically active. The drugs are commonly used in combination to improve efficacy as part of the highly active antiretroviral therapy (HAART) regimen. The need for combination therapy has led to the development of multiplexed assays to measure many of the prescribed drugs in one run [29–34]. The combinations of drugs within these methods need to be wide because it is common practice to switch drugs during therapy especially when viral resistance is encountered. HIV drugs are typically stored frozen until analysis which is

Table 1
LC–MS/MS methods for the quantification of antifungal drugs.

Drug	Sample volume	Internal standard	Sample preparation	Ionisation mode	Column	LLOQ	Linearity	Reference
Voriconazole	10 μL	Ketoconazole	PP	ESI+	C18	100 μg/L	20,000 μg/L	[12]
Voriconazole	5 μL	None	Online SPE	ESI+	C18	50 μg/L	5000 μg/L	[13]
Posaconazole	100 μL	SCH56984	PP	APCI+	C18	5 μg/L	5000 μg/L	[18]
Posaconazole	50 μL	$^{15}N_2{}^{13}C$posaconazole	SPE	ESI+	C18 halo	5 μg/L	5000 μg/L	[17]
Itraconazole and hydroxyitraconazole	500 μL	Fluconazole	SPE	ESI+	C18	0.5 μg/L	263 μg/L	[14]
Iodiconazole	400 μL	Iodiconazole analogue	LLE	ESI+	C18	0.1 μg/L	20 μg/L	[19]
Voriconazole, fluconazole, itraconazole, hydroxyitraconazoleand posaconazole	100 μl	Cyandimipramine	PP	ESI+	C18	100 μg/L	200,000 μg/L	[23]
Itraconazole, voriconazole and posaconazole	200 μL	Quinoxaline	PP	ESI+	C18	30 μg/L	10,000 μg/L	[22]
Voriconazole, fluconazole, posaconazole, itraconazole and hydroxyitraconazole	75 μL	Ketoconazole	PP	ESI+	C18	10 μg/L	10,000 μg/L	[20]
Fluconazole, itraconazole, posaconazole, voriconazole, caspofungin and anidulafungin	100 μL	Deuterated compounds	PP	ESI+	C18 UPLC	10 μg/L	50,000 μg/L	[21]
Anidulofungin, caspofungin, isavuconazole, micafungin, posaconazole and voriconazole	3 μL	Itraconazole	PP	ESI+	C4	4.2 μg/L	Not stated	[24]
Fluconazole, itraconazole, posaconazole and voriconazole	100 μL	Dimethylitraconazole	PP	ESI+	C8	100 μg/L	12,000 μg/L	[25]

fine in most cases but some authors have pointed out the occurrence of losses of about 15% due to the degradation of some thermo-labile compounds such as atazanavir [35].

It has been mentioned that the NTRIs are phosphorylated within the cell and their efficacy is related to the intracellular metabolite concentration. The NTRIs pose particular analytical problems because not only do they need to be measured in peripheral blood mononuclear cells, which is not a straightforward procedure for routine TDM, but they are also highly polar and may need ion pairing reagents to improve chromatographic retention and peak shape [36]. It is recognised that LC–MS/MS is the best detection option for determining HIV drugs and in particular the superior analytical performance makes it possible to measure intracellular sub-ng/mL concentrations of NNRTI, PI drugs, and phosphorylated NRTI metabolites [27].

HIV drugs exhibit wide differences in polarity and this can have a significant effect on the method of sample clean-up used. LLE is good for the extraction of PIs and NNRTIs from plasma but because the NTRIs are very polar they extract poorly with LLE and need SPE or PP procedures. SPE and PP are also good for extracting PIs and NNRTIs from plasma. The majority of LC–MS/MS methods have used PP to extract the samples but combined extracts of both PP and LLE have been used to extract a wider range of drugs [37]. An on line SPE method has been reported that used cimetidine as a single IS with no chromatographic separation of 11 analytes with a fast run time of 4.5 min. It was claimed that ion suppression was minimal because APCI was used and the method was later modified to include atazanavir and tipranavir [33]. A dilution step was included in the sample extraction to account for higher concentrations of TPV seen in some samples.

The most common analytical columns used are C18 although to obtain adequate separation of the more polar compounds, particularly TND, zalcitabine and lamivudine, some methods have used gradients with initial highly aqueous mobile phase composition. The LC columns used in these situations are by necessity highly end-capped and therefore designed to operate under highly aqueous conditions [37–40]. Electrospray is the most common ionization source and detection is usually performed in positive mode. The exceptions to this are the thymine analogues such as zidovidine and the NTRIs didanosine, stavudine and zidovudine, which are measured in negative ion mode. This is achieved in some methods whilst still maintaining sensitivity by polarity switching during the run whereby the ionization mode is switched from positive to negative over the short period of time when the relevant analytes elute. [37,38]. Compounds such as didanosine and efavirenz can be measured in both positive and negative ion mode.

A variety of internal standards have been used ranging from structural, analogues in the earlier methods to isotopically labelled compounds. Stable isotopes are thought to be important for the measurement of chemically similar compounds which are co-administered, e.g. tenofovir and emtricitabine [41].

Detection limits depend on the sensitivity of the instrument and the amount of sample extracted, but are in the order of magnitude of 0.1–10 ng/mL.

Measurement of HIV drugs is possible in other sample matrices including semen, breast milk, hair, CSF, ultrafiltrate and PBMC. The measurement of free or unbound drug may be more appropriate for TDM of some drugs because many of the PI and NTRIs are highly protein bound [42]. Methods have been developed using ultrafiltration devices to separate the free fraction which can then be measured by LC–MS/MS [43,44], but their application in a routine setting has yet to be established.

Antiviral drugs are also used in the treatment of hepatitis C and cytomegalovirus, which can be an especial problem in immunocompromised patients. These drugs include ribavirine, used to treat hepatitis C, and ganciclovir, used in the prevention and treatment of CMV. TDM of these drugs is not routinely used, but may be of use if there is a question of patient compliance or there are symptoms of toxicity. Ganciclovir may be prescribed in its active form or as the prodrug, valganciclovir. Similarly, ribavirin may be given as the prodrug viramidine. It is therefore advantageous to be able to quantify both forms of the drug simultaneously, and LC–MS/MS assays have been developed for both groups of drugs to facilitate this. Protein precipitation is the sample preparation method of choice for all these drugs [45–49], although Singh et al. used SPE with MCX cartridges to clean up plasma samples prior to quantification of ganciclovir and valganciclovir [50]. Ganciclovir and valganciclovir can be separated using a C18 [50] or a silica column [49], but ribavirin and viramidine are very polar molecules, and a column such as hypercarb [45], or ODS-BP [47] may be useful to increase retention times and facilitate chromatographic separation from isobaric interferents such as uridine and cytosine [45] (Table 2).

4. Immunosuppressant drugs

The application of LC–MS/MS to this group of drugs has been comprehensively reviewed by Yang [54]. The immunosuppressant drugs that are routinely monitored consist of three main classes the calcineurin inhibitors (CI) ciclosporin (CsA) and tacrolimus (TAC), the mTor inhibitors sirolimus (SIR) and everolimus (Evero), and the inosine monophosphate dehydrogenase inhibitor mycophenolic acid (MPA).

The calcineurin inhibitors and mTor inhibitors freely partition into red blood cells so the preferred sample type is whole blood with EDTA as the preferred anticoagulant [55] whereas the preferred sample type for MPA is plasma. The CI inhibitors and mTor inhibitors ionize readily in ES positive ion mode but they undergo poor fragmentation resulting in low abundance of product ions. These drugs do however form adducts in the source with ionic mobile phase modifiers such as ammonium, sodium, potassium and caesium. The most popular adduct used for analysis is the ammonium adduct although there has been one published method using caesium [56], although this method was not fully worked up and only qualitative data was presented. Potassium and sodium tend to form more stable adducts which can be difficult to fragment in some instruments, although sodium has been used for single ion monitoring. Sodium in particular can cause sensitivity problems in routine methods using ammonium adduct formation because of preferential adduct formation, the contamination can arise from solvents or source contamination with sample matrix. It has been reported that APCI sources operating in negative ion mode suffer less with ion suppression than ESI in positive ion mode for the analysis of immunosuppressant drugs [57], but the most popular methods are still those using fragmentation of ammonium adducts in electrospray positive ion mode. The first reported methods using LC–MS/MS were derived from HPLC-UV methods with long and cumbersome sample clean up steps such as off line SPE and LLE. It was quickly discovered that the specificity of LC–MS/MS allowed the preparation of crude sample extracts with PP and rapid chromatography using short analytical columns and ballistic gradients [58,59]. These methods achieved run times of less than 3 min thus allowing the rapid reporting of test results within several hours, importantly the rapid turnaround of results made these assays a viable alternative to immunoassay. The commonest sample prep is PP using a combination of aqueous zinc sulphate and acetonitrile and this was later modified to improve sample extraction quality [60]. With the introduction of more sophisticated instruments possessing faster scan speeds it has become possible to analyse more than one analyte in a single run and this has permitted the development of multiplexed assays for the measurement of several analytes simultaneously. It is possible to prescribe an mTor

Table 2
LC–MS/MS methods for the measurement of antiviral drugs.

Drug	Sample volume	Internal standard	Sample preparation	Ionisation mode	Column	LLOQ	Linearity	Reference
Nelfinavir, indinavir, ritonavir, saquinavir and amprenavir	100 μL	Methyl indinavir	PP	ESI+	C8	5 μg/L	5–10,000 μg/L	[29]
Zalcitabine, 2′,3′-dideoxythymidine, 3′-azido-3′-deoxythymidine, indinavir, abacivir, nelfinavir, saquinavir, nevirapine, lamivudine, ritonavir and lopinavir	80 μL	Cimetidine	PP and online SPE	ESI+ and −	C18	2 μg/L	10,000 μg/L	[51]
Nelfinavir, indinavir, ritonavir, saquinavir, amprenavir, lopinavir and M8	250 μL	A86093	LLE	ESI+	C18	8 μg/L	10,000 μg/LL	[30]
Amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir and saquinavir	100 μL	Ro31-9564	PP	ESI+	C18	25 μg/L	15,000 μg/L	[32]
Efavirenz, nelfinavir, nevirapine, saquinavir, zalcitabine, amprenavir, zidovudine, atazanavir, delavirdine, indinavir, lopinavir and ritonavir	200 μL	d5-Saquinavir, cyclospropyl ritonavir and S-CH3-saquainvir	PP	ESI+ and −	C18	10 μg/L	20,000 μg/L	[38]
Efavirenz, nevirapine, zidovudine, stavudine, abacivir, lamivudine, zalcitabine, didanosine, indinavir, nelfinavir, ritonavir, atazanavir, saquinavir, lopinvair andamprenavir	50 μL	Cimetidine	LLE and PP	ESI+ and −	C18	1 μg/L	500 μg/L	[37]
Amprenavir, atazanavir, efavirenz, indinavir, lopinavir, nelfinavir, nevirapine, ritonavir, saquinavir and tipranavir	100 μL	d5-Saquinavir, d6-indonavir, ^{13}C-efavirenz and dibenzipine	LLE	ESI+	C18	50 μg/L	20,000 μg/L	[52]
Abacivir, lamivudine, zidovudine, tenofovir, emtricitabine, didanosine and stavudine	50 μL	6β-Hydroxy-theophylline	PP	ESI+	C18	10 μg/L	4000 μg/L	[39]
Darunavir, etravirine, maraviroc, raletegravir and ritonavir	100 μL	d9-Darunavir	PP	ESI+	C18	5 μg/L	10,000 μg/L	[40]
Emtricitabine and tenofovir	250 μL	$^{13}C^{15}N$-isoemtricitabine and ^{13}C-isotenofovir	PP	ESI+	C18	10 μg/L	1500 μg/L	[41]
Amprenavir, atazanavir, lopinavir, ritonavir, nevirapine, darunavir, etravirine and rilpirivine	100 μL	Quinoxalone	PP	ESI+	C18	5 μg/L	15,000 μg/L	[34]
Amprenavir, lopinavir, ritonavir, saquinavir, tipranavir	8 mL	A86093	PBMC extraction	ESI+	C18	2 ng/3 × 10^6 cells	200 ng/3 × 10^6 cells	[31]
Amprenavir, atazanvir, efavirenz, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir, tipranavir	8 mL	d5-Atazanavir, d6-indinavir, d5-saquinavir, ^{13}C-ritonavir, d8-lopinavir, d4-efavirenz	PBMC extraction	ESI+ and −	C18	0.05 μg/L	125 μg/L	[53]
Ribavirin	100 μL	$^{13}C_5$-ribavirin	PP	ESI+	Hypercarb	50 μg/L	5000 μg/L	[45]
Ribavirin	100 μL	Bamethan	PP	ESI+	Silica	10 μg/L	10,000 μg/L	[46]
Ribavirin	100 μL	Acyclovir	PP	ESI+	ODS-BP	1 μg/L	1000 μg/L	[47]
Ribavirin and viramidine	100 μL	^{13}C-ribavirine, ^{13}C-viramidine	PP	ESI+	C18	1 μg/L	1000 μg/L	[48]
Ganciclovir (G) and valganciclovir (V)	10 μL	d5-Ganciclovir and d5-valganciclovir	PP	ESI+	Silica	16 μg/L (G) 4 μg/L (V)	40,000 μg/L (G) 10,000 μg/L (V)	[49]
Ganciclovir and valganciclovir	200 μL	Acyclovir andvalaciclovir	SPE	ESI+	C18	70 μg/L (G) 5 μg/L (V)	11,200 μg/L (G) 800 μg/L (V)	[50]

inhibitor with a CI but the reason for measuring all of the drugs simultaneously is to make sample handling easier. Some methods perform direct injection of the supernatant from the protein crash [57,61,62], whilst others use on line SPE to further purify the sample [63–65]. Analytical sensitivity is not an issue with these drugs and all methods can be operated on modern entry level instruments which generally have sufficient scan speeds to support multiplexed assays. CsD has been the most popular IS for CsA but this is now being replaced by deuterated CsA because of possible interference in the IS transition [8,66]. Deuterated tacrolimus is also available but ascomycin is still commonly used and appears to perform well despite the drawback of its variable stability when dissolved in acetonitrile as part of the protein crash reagent [67]. The stability of ascomycin has been reported to be from as little as several hours in some cases to several months. Ascomycin has also been widely used as an IS for everolimus and sirolimus although again this is now being replaced by deuterated compounds which are now available commercially. The influence of matrix effects on IS assays has been further investigated by Vethe et al. [68]. They found that the elution of matrix components including glycerophosphocholines overlapped to some extent with the target compounds, and the average ion suppression ranged from 8.5 to 21%. However, the drugs and internal standards were influenced to the same extent and they concluded that the internal standards consistently corrected for the between-individual variability of matrix effects. There are now several commercially available assays with IVD-CE certification for the measurement of immunosuppressant drugs. These suppliers also provide separate calibrator and quality control reagents. Chromsystems (Chromsystems Instruments & Chemicals GmbH) and Recipe® (Recipe Chemicals & Instruments GmbH, Germany) provide a 4 in 1 assay combining ciclosporin, tacrolimus, everolimus and sirolimus. After an initial protein crash, the analytes are concentrated on a trap column before separation on the analytical column. (www.chromsystems.de, www.recipe.de). The Chromsystems kit uses isotopically labelled internal standards, whereas the Recipe® kit uses deuterated everolimus with ascomycin and ciclosporin D. Both of these kits are designed to work with any manufacturer's mass spectrometer, whereas the Waters MassTrak™ Immunosuppressants XE kit (Waters Corporation, USA) is fully certified to work only with that manufacturer's equipment (www.waters.com). The Waters MassTrak™ Immunosuppressants XE kit is currently only available for measuring tacrolimus and everolimus using ascomycin and isotopically labelled everolimus as internal standards. All three suppliers claim analytical run times of less than 2 min per sample.

5. Anticonvulsants

Anticonvulsant drugs are used for the control of partial and full epileptic seizures. The use of multiple drugs is often necessary to prevent seizures occurring; however interactions between antiepileptics affect levels of these drugs in the circulation. Carbamazepine often lowers plasma levels of other anticonvulsants such as lamotrigine and valproate, whereas valproate often increases levels of lamotrigine and phenytoin. Therapeutic drug monitoring of anticonvulsants is therefore very important especially when patients are being maintained on multiple drugs. Toxic levels of some anticonvulsants, especially valproate and phenytoin, can lead to seizures, so therapeutic drug monitoring can be very useful in a convulsing patient to ascertain whether their symptoms are due to ineffectively low or toxic levels of their anticonvulsant. Anticonvulsant measurement is generally carried out by automated immunoassay, as results are often required urgently to inform patient management. LC–MS/MS methods have been published for many of the anticonvulsants, and may provide advantages in terms of specificity and sensitivity of measurement, and also some methods can quantify multiple drugs, which may be beneficial as patients are often prescribed more than one anticonvulsant.

The majority of the anticonvulsants can be quantified using positive ionisation mode and a C18 column. Isotopic internal standards are preferred as they behave in a similar fashion to the drug of interest and can compensate for variations in ionisation, however these are not always commercially available and it may be necessary to use alternatives such as structural analogues, e.g. metformin in gabapentin quantification [69,70] or ritonavir for levetiracetam measurement [71,72]. A variety of sample preparation methods have been employed to clean up samples prior to anticonvulsant measurement. Anticonvulsants are present in relatively high concentrations in plasma so protein precipitation is generally sufficient for their quantification, LLE [73,74] or SPE [75] may be necessary if quantification of metabolites or multiple drugs is required.

Gabapentin, carbamazepine oxcarbazepine and valproate are the drugs of choice for treatment of focal seizures. The majority of these drugs are ionised in positive mode. The exception to this is valproate which is an acid so ionises much better in negative mode. A C18 analytical column is commonly used to separate these analytes; however gabapentin is a polar molecule and sensitivity may be improved by using a C4, C8, or HILIC column to improve retention on the column. Many of the published methods also quantify metabolites of these drugs. This may be a problem with oxcarbazepine, as the main metabolite, 10-hydroxyoxcarbazepine, may fragment to form parent and daughter ions of the same mass as the parent drug; full chromatographic separation is therefore required to avoid analytical interference.

Lamotrigine, levetiracetam and phenytoin are commonly prescribed for the treatment of seizures. LC–MS/MS methods for these drugs generally use a C18 column with the mass spectrometer in positive ionisation mode. Subramanian et al. developed a method for the simultaneous quantification of 9 anticonvulsants including lamotrigine, phenytoin phenobarbital and topiramate [76] using solid phase extraction with Strata-X cartridges and separation on a C18 column. Constant polarity switching was used as 4 of the analytes ionised in negative mode and 5 in positive ion mode. Such a method requires a mass spectrometer with a high scan speed to allow collection of sufficient data points to characterise the peaks of all the analytes and allow optimal peak integration. The run time of this assay was 20 min per sample, which may not be suitable if analysis of a high number of samples is required, however simultaneous quantification of 9 commonly prescribed anticonvulsants may avoid the need to analyse one patient sample on multiple assays, enabling results to be available in a more timely manner.

Ethosuximide, phenobarbital and topiramate are anticonvulsant drugs which can be easily ionised in negative mode. Topiramate can also be ionised in positive ion mode, but sensitivity is increased if negative ionisation is used [77–82]. A C18 analytical column is commonly used for quantification of these drugs. Primidone is an anticonvulsant which is metabolised to phenobarbital, so is essentially a prodrug. Primidone can be analysed by LC–MS/MS using a molecularly imprinted polymer column to selectively introduce primidone into the mass spectrometer [83]. These columns are very expensive and time consuming to produce so unlikely to be adopted for routine use; as phenobarbital is the active metabolite, it is more informative to measure phenobarbital levels for therapeutic drug monitoring purposes (Table 3).

6. Antidepressants

Antidepressant drugs are used in the treatment of moderate to severe depression. Major classes of antidepressants include tricyclics, monoamine oxidase inhibitors and selective serotonin

Table 3
Details of LC–MS/MS methods for the measurement of anticonvulsants.

Drug	Sample volume	Internal standard	Sample preparation	Ionisation mode	Column	LLOQ	Linearity	Reference
Gabapentin	200 μL	Metformin	Protein ppt	ESI	C8	50 μg/L	5000 μg/L	[69]
Gabapentin	10 μL	Metformin	Protein ppt	ESI+	HILIC	50 μg/L	10,000 μg/L	[70]
Gabapentin	100 μL	1,1,cyclo-Hexane diacetic acid monoamide	Protein ppt	API+	C18	40 μg/L	10,000 μg/L	[84]
Gabapentin	200 μL	(*S*)-(α)-amino-cyclohexane-propionic acid hydrate	Protein ppt	API+	C8	7.5 μg/L	135 μg/L	[85]
Gabapentin	100 μL	(*S*)-(α)-amino-cyclohexane-propionic acid hydrate	Protein ppt	ESI+	C18	20 μg/L	5000 μg/L	[86]
Gabapentin	200 μL	Acetaminophen	Protein ppt	ESI+	C4	50 μg/L	10,000 μg/L	[87]
Carbamazepine	300 μL	2-Methyl carbamazepine	Protein ppt	ESI+	C8	500 μg/L	Quadratic curve	[88]
Carbamazepine	500 μL	Nitrazepam	Liquid–liquid extraction	ESI+	C18	0.722 μg/L	10,600 μg/L	[73]
Ethosuximide	250 μL	Pravastatin	SPE	ESI−	C18	250 mg/L	60,000 mg/L	[89]
Lamotrigine	100 μL	^{13}C2, ^{15}N-LTG	SPE	APCI+	Shimpack OR-ODS	0.3125 mg/L	25 mg/L	[76]
Lamotrigine	200 μL	3,5-Diamino-6-(2-methoxyphenyl)-1,2,4-triazine	Protein ppt	ESI+	C18	0.07 mg/L	2.56 mg/L	[90]
Levetiracetam	50 μL	UCB17025	Protein ppt	ESI+	Acquity BEH	0.5 mg/L	150 mg/L	[91]
Levetiracetam	50 μL	Ritonavir	Protein ppt	ESI+	C18	1 mg/L	50 mg/L	[71]
Levetiracetam	100 μL	Adenosine	SPE	ESI+	C18	1 mg/L	40 mg/L	[92]
Levetiracetam	100 μL	Ritonavir	Protein ppt	ESI+	C18	0.1 mg/L	50 mg/L	[72]
Levetiracetam	200 μL	Clonazepam	SPE	ESI+	C18	0.5 mg/L	50 mg/L	[93]
Oxcarbazepine	Not stated	Imipramine	Liquid–liquid extraction	ESI+	C18	0.2 mg/L	16 mg/L	[94]
Oxcarbazepine	150 μL	Cyheptamide	SPE	ESI+	C18	0.078 mg/L	5 mg/L	[75]
Oxcarbazepine	100 μL	d10-Carbamazepine	Liquid–liquid extraction	ESI+	C18	0.02 mg/L	5.25 mg/L	[74]
Oxcarbazepine	200 μL	d3-Trimipramine	Liquid–liquid extraction	APCI+	Superspher 60RP select B	0.1 mg/L	5 mg/L	[95]
Phenobarbital	100 μL	*o*-Acetamidophenol	SPE	ESI−	C18	1 mg/L	100 mg/L	[96]
Phenytoin	50 μL	Phenacetin	Protein ppt	ESI+	C18	0.01 mg/L	25 mg/L	[97]
Free phenytoin	1000 μL	Phenobarbital	Liquid–liquid extraction	APCI−	Inertsil ODS-3	0.005 mg/L	0.5 mg/L	[98]
Primidone	500 μL	None	Molecular imprinted polymer	ESI-IMS	Direct injection	0.02 mg/L	2 mg/L	[83]
Topiramate	100 μL	d12-Topiramate	Liquid–liquid extraction	ESI−	C18	0.5 mg/L	30 mg/L	[77]
Topiramate	300 μL	Amlodipine	SPE	TSI−	C18	0.0104 mg/L	2.05 mg/L	[78]
Topiramate	100 μL	Prednisone	Protein ppt	ESI−	C18	0.2 mg/L	5.0 mg/L	[79]
Topiramate	500 μL	d12-Topiramate	Liquid–liquid extraction	ESI+	C18	0.625 mg/L	40 mg/L	[80]
Topiramate	200 μL	1,2:3,4-bis-*o*-(1-Methylethylidene-α-D-galactopyranose sulfamate	Protein ppt	API−	C18	0.02 mg/L	20 mg/L	[81]
Topiramate	500 μL	None	Protein ppt	TIS+	C18	1 mg/L	20 mg/L	[82]
Valproate	200 μL	Betamethasone valerate	SPE	ESI−	C18	0.5 mg/L	150 mg/L	[99]
Valproate	200 μL	Benzoic acid	SPE	API−	C18	0.5 mg/L	60 mg/L	[100]
Valproate	20 μL	None	SPE	API−	ShimPack CLC-ODS	5 mg/L	1000 mg/L	[101]
Pregabilin	100 μL	Rosuvastatin	Liquid–liquid extraction	APCI+	C18	0.001 mg/L	10 mg/L	[102]

re-uptake inhibitors. Therapeutic drug monitoring of antidepressants is not routinely required, but may be useful if non-adherence to medication regimes or an overdose is suspected, which may be a fairly frequent occurrence, especially in an outpatient setting.

Amitriptyline, doxepin and nortriptyline are tricyclic antidepressants; mianserin and trazodone are tricyclic-related drugs. These drugs can all be measured by LC–MS/MS in positive ionisation mode with a C18 analytical column. Use of a cyano column enables the chromatographic separation of trazodone and its main metabolite, *m*-chlorophenylpiperazine, and the use of a high ion voltage increases the ionisation efficiency of the drug [103].

Monoamine oxidase inhibitors are used as second line treatment for depression. Moclobemide is a reversible MAOI used in the treatment of severe depression and social anxiety disorder. This drug can quantified by LC–MS/MS following solid phase extraction using C18 cartridges followed by separation on a phenyl column [104]. Electrospray positive ionisation was used, although ionisation can also be achieved using APCI.

Selective serotonin reuptake inhibitors such as citalopram, escitalopram, fluoxetine, paroxetine and sertraline are often used as first line treatment for depression. These drugs are safer than many of the other antidepressants, especially if an overdose is taken, so therapeutic drug monitoring is rarely required unless non-compliance with treatment is suspected. The majority of these drugs can be analysed using a C18 column with the mass spectrometer in electrospray positive mode, apart from sertraline, a polar molecule which shows better retention using a C8 column [105,106], and paroxetine, which has been shown to have better chromatographic separation and more efficient ionisation using a HILIC column with a highly organic mobile phase containing 0.05% TFA [107]. It has been hypothesised that the enantiomers of fluoxetine may exhibit different therapeutic effects and be metabolised at different rates. Shen et al. developed a method to separate and quantify the enantiomers of fluoxetine using automated LLE with ethyl acetate and a chirabiotic V chiral column [108]. Measurement of fluoxetine or its enantiomers is not routinely required for therapeutic drug monitoring purposes, but such an assay may prove useful for pharmacokinetic studies. The enantiomers of mirtazepine, a serotonergic and noradrenergic antidepressant can also be separated and quantified using LLE followed by separation using a chiral column [109], but this technique is very time-consuming and unsuitable and indeed unnecessary for routine use in TDM.

Atypical antipsychotics are better tolerated than the older antipsychotic drugs and cause fewer extrapyramidal side effects. Therapeutic drug monitoring is therefore rarely required. Drugs in this class include amisulpride, aripiprazole, clozapine, olanzapine, quetiapine and risperidone. As with the other antipsychotic drugs, the majority can be quantified by LC–MS/MS in positive ionisation mode using a C18 column. Liquid–liquid extraction is the sample preparation method of choice for this class of drugs due to the low levels found in plasma [110–119], SPE may also be useful [120–123] but protein precipitation is unlikely to provide the necessary sensitivity to allow measurement of the atypical antipsychotics in serum (Table 4).

7. Antibiotics

Different classes of antibiotics work in different ways to treat bacterial infections. Antibiotics are widely prescribed, but problems with organisms developing resistance to these drugs means that their efficacy may be lost and care should be taken to avoid unnecessary prescription. Some of the drugs such as the penicillins have a wide therapeutic index, so measurement of plasma levels is rarely necessary. Other classes such as the aminoglycosides have dose-related toxic effects and therapeutic drug monitoring is often used when patients are prescribed these drugs.

Amoxicillin is a broad spectrum penicillin often used in the treatment of inner-ear infections and exacerbation of chronic bronchitis. It is often prescribed with the β-lactamase inhibitor clavulanic acid to prevent bacterial resistance to the drug. Amoxicillin is a polar molecule, so use of a column such as C8 or HILIC is required to improve retention of the drug [155]. As the therapeutic index of amoxicillin is so wide, therapeutic drug monitoring is not generally required.

The aminoglycoside antibiotics include amikacin, tobramycin, gentamicin, streptomycin, and neomycin. These drugs are not effectively absorbed by the gut, so must be administered intravenously for systemic infections. Tobramycin is used for the treatment of *Pseudomonas aerunigosa* lung infections so can be delivered by nebuliser directly to the affected organ. Side effects are dose related and include nephro- and ototoxicity; these most often occur in patients with renal impairment as the aminoglycosides are renally excreted. Vancomycin is a glycopeptide antibiotic with a similar toxic effect profile. Therapeutic drug monitoring of the aminoglycosides and vancomycin is often carried out by immunoassay as results may be required quickly to allow the next dose of the drug to be given, but LC–MS/MS methods have been published for the quantification of tobramycin, neomycin and vancomycin. Both drugs can be measured in electrospray positive ionisation mode, with protein precipitation used as sample preparation. Tobramycin can be easily separated using a C18 column [156], but neomycin and vancomycin are very polar molecules and use of a HILIC or C8 column may improve retention of this molecule [157–159].

Tuberculosis is treated in two phases using multiple drugs in each stage, to try and prevent the emergence of drug resistant bacteria. Isoniazid, rifampicin, pyrazinamide and ethambutol are used in the initial phase, with isoniazid and rifampicin used together in the continuation phase. Therapeutic drug monitoring is not recommended for these drugs, but may become necessary if overdose or non-compliance is suspected. These drugs can be measured by LC–MS/MS with a simple protein precipitation step used for sample preparation. All can be ionised in positive mode, and C18 is the most popular analytical column used. Many of the methods published can quantify multiple drugs simultaneously [160–162], which is advantageous when patients are being treated with several drugs.

Cephalosporins are broad spectrum antibiotics which can be used in the treatment of pneumonia, septicaemia, meningitis and other infections. The cephalosporins are a subclass of the β-lactam antibiotics, and include drugs such as cephalexin, cefixime, cefuroxime and cefoperazone. C18 is the most popular analytical column used for the cephalosporins, and they contain both amino and carboxylic acid groups, so can ionise in both negative and positive mode [163–167].

Colistin is a polymyxin antibiotic used against gram negative bacteria. Dose-related side effects include neuro and nephrotoxicity, therapeutic drug monitoring of this drug may therefore be useful. Colistin consists of two structurally related decapeptides, colistin A and colistin B, which differ only by the fatty acid chain connected to the tail of the decapeptide. The colistins can be ionised in both positive [168] and negative [169] mode. C18 is the analytical column of choice, and use of an internal standard such as polymyxin B seems to widen the analytical range.

Other antibiotics commonly prescribed include sulphonamides such as sulfamethoxasole and trimethoprim, metronidazole, clindamycin, quinolones such as levefloxacin, the macrolide antibiotics azithromycin and clarithromycin, and the lipopeptide antibiotic daptomycin. These drugs do not meet the requirements for therapeutic drug monitoring, but LC–MS/MS methods have been published describing their quantification for pharmacokinetic

Table 4
Details of LC–MS/MS methods for the measurement of antidepressants.

Drug	Sample volume	Internal standard	Sample preparation	Ionisation mode	Column	LLOQ	Linearity	Reference
Amisulpride	200 μL	d5-Amisulpride	Liquid–liquid extraction	APCI+	Spherisorb S5SCX	0.5 μg/L	150 μg/L	[111]
Amisulpride	100 μL	Eticlopride	Liquid–liquid extraction	ESI+	C18	0.1 μg/L	500 μg/L	[110]
Aripiprazole	500 μL	Papaverine	Liquid–liquid extraction	ESI+	Phenyl	1 μg/L	600 μg/L	[112]
Aripiprazole	400 μL	OPC 14714	Liquid–liquid extraction	ESI+	C18	0.1 μg/L	100 μg/L	[113]
Citalopram	200 μL	Desipramine	Protein ppt	ESI+	C18	0.0002 mg/L	0.1 mg/L	[124]
Citalopram	500 μL	Imipramine	Liquid–liquid extraction	ESI+	C8	0.5 μg/L	250 μg/L	[125]
Clozapine	50 μL	Mirtazepine	SPE	ESI+	C18	10 μg/L	1000 μg/L (non-linear regression used)	[126]
Clozapine	500 μL	Congener of risperidone	Liquid–liquid extraction	ESI+	C18	1 μg/L	1000 μg/L	[114]
Doxepin	500 μL	Benzoctamine-HCl	Liquid–liquid extraction	ESI+	C18	0.3 μg/L	81 μg/L	[127]
Duloxetine	200 μL	Haloperidol	Protein ppt	API+	C18	0.1 μg/L	50 μg/L	[128]
Escitalopram	100 μL	Paroxetine	Liquid–liquid extraction	ESI+	ODS YMC AQ (C18)	1 μg/L	200 μg/L	[129]
Fluoxetine	500 μL	Metronidazole	Liquid–liquid extraction and SPE	ESI+	C18	5 μg/L	40 μg/L	[130]
Fluoxetine	Not stated	Methylfluoxetine	SPE	ESI+	C18	25 μg/L	1000 μg/L	[131]
Fluoxetine	200 μL	d2-Fluoxetine	Liquid–liquid extraction	ESI+	C18	0.1 μg/L	22 μg/L	[132]
Fluoxetine	200 μL	Oxazepam	Liquid–liquid extraction	APCI+	Chirobiotic V	2 μg/L	1000 μg/L	[108]
Fluoxetine	500 μL	Desipramne	Liquid–liquid extraction	ESI+	C18	0.15 μg/L	5 μg/L	[133]
Flupentixol	500 μL	Mosapride	Liquid–liquid extraction	ESI+	C8	2.6 μg/L	2090 μg/L	[134]
Haloperidol	2000 μL	Chlorohaloperidol	Liquid–liquid extraction	ESI+	C18	0.1 μg/L	50 μg/L	[135]
Mianserin	200 μL	Cinnarizine	Liquid–liquid extraction	ESI+	C18	1 μg/L	200 μg/L	[136]
Mirtazepine	1000 μL	Haloperidol	Liquid–liquid extraction	ESI+	Chiralpak AD-RH	0.125 μg/L	125 μg/L	[109]
Moclobemide	500 μL	Ro11-9900	SPE	ESI+	Phenyl	10 μg/L	520 μg/L	[104]
Nortriptyline	1000 μL	d4-Nortriptyline	Liquid–liquid extraction	APCI+	C18	0.8 μg/L	32 μg//L	[137]
Olanzapine	50 μL	d3-Olanzapine	Liquid–liquid extraction	ESI+	Xbridge Shield RP	0.1 μg/L	30 μg/L	[115]
Olanzapine	500 μL	Loratadine	Liquid–liquid extraction	ESI+	C18	0.1 μg/L	30 μg/L	[116]
Olanzapine	250 μL	LY170158	Liquid–liquid extraction	APCI+	Metachem Monochrom	5 μg/L	500 μg/L	[117]
Olanzapine	100 μL	2-Ethyl-4-(4-methyl-1-piperazinyl)-10H-thieno[2,3-b] [1,5]benzo-diazepine)	SPE	APCI+	C18	1 μg/L	100 μg/L	[120]
Olanzapine	500 μL	LY170222	SPE	APCI+	MetaChem MonoChrom	0.25 μg/L	50 μg/L	[121]
Paroxetine	400 μL	d5-Fentanyl	Liquid–liquid extraction	ESI+	HILIC (Betasil silica)	0.05 μg/L	50 μg/L	[107]
Paroxetine	1000 μL	Pholedrine	Liquid–liquid extraction	ESI+	Synergi MAX-RP	0.75 μg/L	100 μg/L	[138]
Paroxetine	500 μL	Fluoxetine	Liquid–liquid extraction	ESI+	C18	0.2 μg/L	50 μg/L	[139]
Pimozide	500 μL	Cinnarizine	Liquid–liquid extraction	ESI+	C18	0.02 μg/L	12.8 μg/L	[140]
Prochlorperazine	500 μL	Amitryptiline-HCl	Liquid–liquid extraction	ESI+	C18	0.2 μg/L	6.4 μg/L	[141]
Quetiapine	40 μL	^{13}C6-quetiapine	Liquid–liquid extraction	ESI+	C18	0.5 μg/L	500 μg/L	[118]
Quetiapine	100 μL	Clozapine-HCl	Liquid–liquid extraction	ESI+	C8	0.25 μg/L	500 μg/L	[119]
Quetiapine	500 μL	Clozapine	SPE	ESI+	C18	1 μg/L	382 μg/L	[122]
Risperidone	200 μL	d2-^{13}C2-risperidone	SPE	ESI+	Chiralcel OJ	0.2 μg/L	100 μg/L	[123]
Risperidone	100 μL	Methyl risperidone	Protein ppt	ESI+	C18	0.1 μg/L	15 μg/L	[142]
Risperidone	500 μL	R 68808	Liquid–liquid extraction	ESI+	Phenyl	0.1 μg/L	100 μg/L	[143]
Sertraline	300 μL	Fluoxetine	Liquid–liquid extraction	ESI+	C8	0.5 μg/L	150 μg/L	[105]
Sertraline	250 μL	Diphenhydramine	Liquid–liquid extraction	APCI+	C18	0.1 μg/L	100 μg/L	[144]
Sertraline	475 μL	Imipramine	SPE	ESI+	C8	0.5 μg/L	60 μg/L	[106]
Trazodone	500 μL	Nefazodone	Liquid–liquid extraction	ESI+	Cyano	10 μg/L	3000 μg/L	[103]
Tryptophan	50 μL	d5-Tryptophan	SPE	ESI+	C18	0.5 ng/L	5000 ng/L	[145]
Tryptophan	500 μL	None used	Protein ppt	ESI+	C18	1220 ng/L	19,380,000 ng/L	[146]
Venlafaxine	500 μL	Verapamil	Liquid–liquid extraction	ESI+	C18	0.2 μg/L	200 μg/L	[147]
Venlafaxine	Not stated	Fluoxetine	Liquid–liquid extraction	ESI+	C18	4 μg/L	400 μg/L	[148]
Venlafaxine	200 μL	Nadolol	Protein ppt	ESI+	Cyano	2 μg/L	500 μg/L	[149]
Venlafaxine	1000 μL	Clozapine	Liquid–liquid extraction	ESI+	C18	1 μg/L	200 μg/L	[150]
Venlafaxine	500 μL	Estazolam	Liquid–liquid extraction	ESI+	C18	4 μg/L	700 μg/L	[151]
Venlafaxine	500 μL	Sildenafil	Liquid–liquid extraction	ESI+	Chirobiotic V	1 μg/L	400 μg/L	[152]
Venlafaxine	500 μL	Escitalopram	SPE	ESI+	C18	3 μg/L	300 μg/L	[153]
Zuclopenthixol	1000 μL	Flupenthixol	Liquid–liquid extraction	ESI+	C18	1 μg/L	800 μg/L	[154]

purposes. These antibiotics can be ionised in positive mode, and C18 is the most commonly used analytical column for these drugs. Levofloxacin is a polar molecule, and chromatography of this drug is improved by the use of a column such as HILIC and a highly organic mobile phase to increase retention on the column [170] (Table 5).

8. Anticancer drugs

Cytotoxic drugs are used in the treatment of cancer. These drugs are dangerous to handle as they damage normal tissue as well as cancer cells. Types of chemotherapeutic drugs include alkylating agents, vinca alkaloids, antimetabolites and cytotoxic antibiotic. Prescription of these drugs is limited to oncology specialists, and therapeutic drug monitoring of cytotoxic drugs is not required in the main as a good correlation between plasma levels and efficacy and toxicity has not been established. The exception to this is methotrexate, an antimetabolite drug which acts by inhibiting dihydrofolate reductase, disrupting purine synthesis and preventing cell division. High circulating levels of methotrexate can cause severe myelosuppression, requiring folate rescue using leucovorin. Therapeutic drug monitoring is therefore essential for patients treated with high dose methotrexate. Although TDM is not required for the majority of cytotoxic drugs, LC–MS/MS methods have been developed for the quantification of many of these drugs for pharmacokinetic and research purposes.

Alkylating drugs exert their action by causing miscoding of DNA and preventing cell replication. Ifosfamide, melphalan, busulfan, cyclophosphamide and thiotepa are all alkylating drugs used in chemotherapy. These drugs are mainly quantified using LC–MS/MS in positive ion mode with a C18 analytical column. Ifosfamide contains a chiral centre and is prescribed as a racemic mixture, but the two enantiomers are metabolised by different cytochrome P450 enzymes. It may be advantageous to separate and quantify the two enantiomers to obtain a clear picture of the pharmacokinetics and metabolism of this drug; this can be achieved using SPE with HLB cartridges and chromatography on a Chirabiotic T column [188]. Busulfan is used in preparative regimes for haemopoieitc stem cell transplantation in adults and children. It has a narrow therapeutic range so TDM of this drug may be useful. Methods for quantifying busulfan in plasma by LC–MS/MS have been described [189–193], and Rauh et al. [193] have developed a saliva method as an alternative, less invasive way of quantifying busulfan in paediatric patients. Cyclophosphamide and thiotepa are often co-prescribed for the treatment of advanced breast, ovarian and testicular tumours. A combined assay for the measurement of the two drugs may enable results to be available more quickly and a more efficient service to be provided. De Jonge et al. developed a combined assay for the two drugs using a simple protein precipitation method and a C18 analytical column [194]. This method was also used to quantify 4-hydroxy-cyclopghosphamide, a very unstable metabolite of cyclophosphamide. In order to stabilise the metabolite, it was derivatised with semicarbazide at the time of sample collection.

Antimetabolite drugs such as methotrexate, permetrexed, raltitrexed, tegafur and 5-fluorouracil are used to treat cancer by inhibiting cellular enzymes and preventing cell replication. Methotrexate is the only antimetabolite for which therapeutic drug monitoring is recommended, and this is usually carried out by automated immunoassay as urgent analyses may be required. LC–MS/MS methods have been developed to measure methotrexate and also the other antimetabolites; the majority of methods use electrospray positive ionisation mode and a C18 analytical column. Tegafur is a prodrug of 5-fluorouracil. Both drugs can be measured in a single assay using electrospray negative ionisation mode and a C18 column [195], although it was later discovered that the use of positive ionisation mode may provide greater sensitivity [196].

The vinca alkaloids vinblastine, vincristine and vindesine can be used to treat a variety of malignancies including leukaemias and lymphomas. Other types of cytotoxic drugs used include protein kinase inhibitors such as imatinib and erlotinib, topoisomerase inhibitors, e.g. irinotecan and taxanes such as docetaxel and paclitaxel. Again, the majority of these drugs can be measured by LC–MS/MS in positive ionisation mode using a C18 analytical column. An exception is lapatinib, which contains 5 aromatic rings; a PFP column has been shown to give better retention of this drug than a C18 column [197].

Lenalidomide and thalidomide are immune modulating drugs used in the treatment of multiple myeloma. Both drugs are teratogenic and pregnancy must be avoided during treatment and for at least 1 month after. Samples for thalidomide analysis should be buffered with Sorensen's citrate buffer to prevent spontaneous hydrolysis of the drug; samples are then stable at room temperature for at least 24 h and at −20 °C for significantly longer [198]. C18 analytical columns can be used for both these drugs. Although the structures of these drugs are similar, it has been shown that APCI negative ionisation provides the most sensitivity for thalidomide analysis [198], whereas APCI positive ionisation is preferred for lenalidomide [199].

Procarbazine is used in the treatment of lymphomas and brain tumours. This drug degrades rapidly in alkaline solutions, so the use of high pH reagents should be avoided when quantifying this drug. To avoid this, He et al. used TCA to precipitate plasma proteins, then removed the excess acid using MTBE [200] prior to separation on a C18 analytical column.

Carboplatin is used intravenously for the treatment of advanced lung and ovarian cancer. Myelosuppression is a problem with this drug, but other side effects are less severe than with the other platinum based drugs. TDM of carboplatin may be carried out by measuring plasma levels of platinum using atomic absorption. Intact carboplatin can be quantified by LC–MS/MS, but the recovery of the drug in plasma is very low, at only 58.7%, making it unsuitable for routine clinical use [201]. Tamoxifen is an oestrogen antagonist used to treat patients with oestrogen-receptor positive breast cancer. It may be considered a pro-drug as its metabolites such as 4-hydroxy tamoxifen are more active than the drug itself, so it is important to quantify these metabolites as well as the parent drug. Both tamoxifen and procarbazine can be measured in electrospray positive mode with a C18 analytical column [202,203] (Table 6).

9. Drugs affecting the cardiovascular system

There are many different classes of drugs which exert their actions on the cardiovascular system, including positive inotropes, anti-arrhythmics, diuretics and anti-thrombotic drugs. Therapeutic drug monitoring is not appropriate for the majority of these drugs, as their effects can be easily assessed clinically. There are some, however, with have a narrow therapeutic index or multiple side effects, for which TDM has been useful.

Digoxin is a cardiac glycoside drug used in the treatment of heart failure and arrhythmia. Symptoms of toxicity are similar to those of clinical deterioration, so rapid quantification of digoxin is sometimes required to ensure appropriate treatment. LC–MS/MS methods have been described for the measurement of digoxin. The majority use liquid–liquid extraction [238–240] with MTBE or chloroform, but SPE extraction methods have also been described [241,242], including a 96-well plate SPE method [241] which may help to increase the throughput of the method. C18 is the column of choice, and electrospray positive ionisation is used in all the methods. LC–MS/MS methods are far more specific than immunoassays,

Table 5
Details of methods for the measurement of antibiotics.

Drug	Sample volume	Internal standard	Sample preparation	Ionisation mode	Column	LLOQ	Linearity	Reference
Tobramycin	20 μl	Siscomycin	Protein ppt	ESI+	C18	0.15 mg/L	50 mg/L	[156]
Neomycin	100 μL	Kanamycin	Protein ppt	ESI+	C18	0.2 mg/L	50 mg/L	[158]
Neomycin	500 μL	None	Automated SPE	ESI+	HILIC	0.1 mg/L	5.0 mg/L	[157]
Rifampicin and clarithromycin	10 μL	Cyanoimipramine	Protein ppt	ESI+	C18	0.1 mg/L (R) 0.2 mg/L (C)	10 mg/L (R) 5 mg/L (C)	[160]
Ethambutol and pyrazinamide	50 μL	d4-Ethambutol dihydrochloride (E)and d3-pyrazinamide (P)	Protein ppt	APCI+	SpeedROD RP18e	0.01 mg/L (E) 0.05 mg/L (P)	5 mg/L (E) 25 mg/L (P)	[161]
Isoniazid and Ethambutol	100 μL	Metformin HCl	Protein ppt	APCI+	C18	0.01 mg/L (I and E)	5 mg/L (I and E)	[162]
Isoniazid	100 μL	Nialamide	Protein ppt	ESI+	Hypersil silica	0.05 mg/L	10 mg/L	[171]
Cephalexin	20 μL	Sulindac	MISPE	ESI+	C18	0.3 mg/L	25 mg/L	[163]
Cefixime	500 μL	Cefetamet	Protein ppt	ESI+	C8	0.05 mg/L	8 mg/L	[164]
Cefuroxime	500 μL	Cefoxitin	SPE	ESI−	Lichrospher 60RP select B	0.081 mg/L	15.976 mg/L	[165]
Cefoperazone and sublactam	200 μL	Cefuroxime	Liquid–liquid extraction	ESI−	C18	0.1 mg/L (C) 0.02 mg/L (S)	20 mg/L (C) 4 mg/L (S)	[167]
Cefuroxime	100 μL	Cefotaxime	Protein ppt	ESI−	SB-CN	0.025 mg/L	50 mg/L	[166]
Azithromycin	200 μL	Roxithromycin	Liquid–liquid extraction	ESI+	C18	0.005 mg/L	1 mg/L	[172]
Azithromycin	100 μL	Erythromycin	Protein ppt	ESI+	CN	0.005 mg/L	0.6 mg/L	[173]
Azithromycin	500 μL	Roxithromycin	Liquid–liquid extraction	ESI+	C18	0.001 mg/L	1 mg/L	[174]
Azithromycin	200 μL	Clarithromycin	Liquid–liquid extraction	ESI+	C18	0.002 mg/L	1 mg/L	[175]
Azithromycin	50 μL	d3-Azithromycin	Liquid–liquid extraction	APCI+	C18	0.01 mg/L	0.25 mg/L	[176]
Clarithromycin	25 μL	Roxithromycin	Protein ppt	ESI+	Phenyl-hexyl	0.1 mg/L	5 mg/L	[177]
Clarithromycin	50 μL	Telmisartan	Protein ppt	ESI+	C18	0.0005 mg/L	5 mg/L	[178]
Clarithromycin	300 μL	Roxithromycin	Liquid–liquid extraction	TIS+	C18	0.00295 mg/L	20.016 mg/L	[179]
Erythromycin	200 μL	Diazepam	Liquid–liquid extraction	ESI+	C18	0.0005 mg/L	1.0 mg/L	[180]
Erythromycin	500 μL	Roxithromycin	Protein ppt	ESI+	C18	1 mg/L	10 mg/L	[181]
Vancomycin	200 μL	Atenolol	SPE	ESI+	C8	0.005 mg/L	10 mg/L	[159]
Daptopmycin	225 μL	Lidocaine	Protein ppt	ESI+	C18	0.5 mg/L	100 mg/L	[182]
Colistin A and B	250 μL	Polymyxin B	SPE	ESI+	C18	0.024 mg/L (CA) 0.015 (CB)	6.144 mg/L (CA) 3.856 mg/L (CB)	[168]
Colistin A and B	100 μL	None	Protein ppt	ESI−	C18	0.019 mg/L (CA) 0.01 mg/L (CB)	2.42 mg/L (CA) 1.315 mg/L (CB)	[169]
Amoxicillin and clavulanic acid	200 μL	Terbutaline	Protein ppt	ESI−	C8	0.125 mg/L (A) 0.062 mg/L (C)	8.0 mg/L (A) 4.0 mg/L (C)	[155]
Levofloxacin	20 μL	Ciprofloxacin	Liquid–liquid extraction	ESI+	HILIC	0.01 mg/L	5 mg/L	[170]
Sulfamethoxazole and trimethoprim	250 μL	Benznidazole	SPE	ESI+	C18	0.5 mg/L (S) 0.05 mg/L (T)	60 mg/L (S) 5.0 mg/L (T)	[183]
Clindamycin	500 μL	Lincomycin	Protein ppt	ESI+	C18	0.05 mg/L	15 mg/L	[184]
Clindamycin	200 μL	d(1)-N-ethylclindamycin	Liquid–liquid extraction	ESI+	C18	0.05 mg/L	3.2 mg/L	[185]
Clindamycin	1000 μL	Lincomycin	Protein ppt	APCI+	RP18	0.1 mg/L	4.0 mg/L	[186]
Clindamycin	100 μL	Verapamil	Protein ppt	ESI	C18	0.05 mg/L	20 mg/L	[187]

Table 6
Details of methods for the measurement of anticancer drugs.

Drug	Sample volume	Internal standard	Sample preparation	Ionisation mode	Column	LLOQ	Linearity	Reference
Busulfan	200 μL	d8-Busulfan	LLE	ESI+	Phenyl	5 μg/L	2500 μg/L	[189]
Busulfan	100 μL	d8-Busulfan	PP	ESI+	C18	36.9 μg/L	10,307 μg/L	[190]
Busulfan	200 μL	Structural analogue	LLE	ESI+	C18	5 μg/L	2500 μg/L	[191]
Busulfan	50 μL	1,6-bis-(Methanesulfonyloxy)hexane	LLE	ESI+	Direct injection	123 μg/L	2460 μg/L	[192]
Busulfan	100 μL	d8-Busulfan	SPE	ESI+	C18	10 μg/L	2500 μg/L	[193]
Ifosfamide	100 μL	None	SPE	ESI+	Chirabiotic T	37.5 μg/L	4800 μg/L	[188]
Erlotinib, gefitinib and imatinib	100 μL	d8-Imatinib	Liquid–liquid extraction	ESI+	C18	5 μg/L (E, G and I)	3000 μg/L (E, G) 5000 μg/L (I)	[204]
Imatinib	200 μL	d8-Imatinib	Liquid–liquid extraction	ESI+	C18	10 μg/L	4000 μg/L	[205]
Imatinib, dasatinib and nilotinib	250 μL	Quinoxaline	Protein ppt	ESI+	C18	78 μg/L (I) 62.5 μg/L (D, N)	10,000 μg/L (I, D, N)	[206]
Imatinib	100 μL	d8-Imatinib	Protein ppt	ESI+	HILIC	1 μg/L	5000 μg/L	[207]
Imatinib	100 μL	d8-Imatinib	Liquid–liquid extraction	ESI+	C18	1 μg/L	5000 μg/L	[208]
Irinotecan	100 μL	Camptothecin	Protein ppt	ESI+	C18	5 μg/L	2000 μg/L	[209]
Irinotecan	50 μL	Camptothecin	Protein ppt	ESI+	C18	1.5 μg/L	100 μg/L	[210]
Irinotecan	200 μL	Camptothecin	Protein ppt	ESI+	C18	2.5 μg/L	10,000 μg/L	[211]
Lapatinib	100 μL	d6-Lapatinib	SPE	APCI+	CuroSil-PFP	1.5 μg/L	10,000 μg/L	[197]
Lenalidomide and flavopiridol	350 μL	Genistein	Protein ppt	APCI+	C18	0.26 μg/L (L) 0.12 μg/L (F)	259.3 μg/L (L) 401.8 μg/L (F)	[199]
Melphalan	40 μL	N-phenyldiethanolamine	Protein ppt	ESI+	C18	1 μg/L	500 μg/L	[212]
Methotrexate	1000 μL	Methotrexate-gamma-(2-hydroxy)ethyl-amide	Diafiltration	FD	C18	0.2 μg/L	30 μg/L	[213]
Mycophenolate	100 μL	N-phthaloyl-L-phenylalanine	Liquid–liquid extraction	ESI+	C18	9 μg/L	100,000 μg/L	[214]
Mycophenolate	500 μL	Indomethacin	SPE	ESI+	C18	50 μg/L	50,000 μg/L	[215]
Nilotinib	200 μL	[^{13}C2,^{15}N2]-nilotinib	Protein ppt	ESI+	Synergi-hydro	5 μg/L	5000 μg/L	[216]
Paclitaxel	200 μL	^{13}C6-paclitaxel	Liquid–liquid extraction	ESI+	C18	100 μg/L	20,400 μg/L	[217]
Paclitaxel	100 μL	d5-Paclitaxel	Liquid–liquid extraction	ESI+	C8	20 μg/L	2500 μg/L	[218]
Docetaxel and paclitaxel	250 μL	Cephalomannine	Liquid–liquid extraction	ESI+	C18	0.4 μg/L	100 μg/L	[219]
Paclitaxel	200 μL	^{13}C6-paclitaxel	Liquid–liquid extraction	ESI+	C18	0.25 μg/L	100 μg/L	[220]
Docetaxel and paclitaxel	250 μL	Cephalomannine	Liquid–liquid extraction	ESI+	C18	2 μg/L	1000 μg/L	[221]
Paclitaxel	100 μL	Paclitaxel analogue	Liquid–liquid extraction	TIS+	C18	1 μg/L	1000 μg/L	[222]
Docetaxel and paclitaxel	1000 μL	Paclitaxel (D) Docetaxel (P)	SPE	ESI+	Hypersil ODS	0.2 μg/L (D) 0.8 μg/L (P)	860 μg/L (D) 850 μg/L (P)	[223]
Paclitaxel	100 μL	None	SPE	ESI−	Direct injection	1 μg/L	1000 μg/L	[224]
Paclitaxel	500 μL	Methyl paclitaxel	SPE	API+	Hypersil ODS	5 μg/L	500 μg/L	[225]
Pemetrexed	500 μL	Dampa	SPE	TIS+	C18	2.5 μg/L	5000 μg/L	[226]
Carboplatin	50 μL	None	SPE	ESI+	ODS Hypersil	70 μg/L	2500 μg/L	[201]
Procarbazine	150 μL	3-Dimethylamino-2-methylpropiophenone	Protein ppt	ESI+	C18	0.5 μg/L	50 μg/L	[200]
Raltitrexed	1000 μL	Benazeprilat	Protein ppt	ESI+	C18	2 μg/L	3000 m/L	[227]
Sorafenib	50 μL	d3-^{13}C-sorafenib	Protein ppt	ESI+	C18	500 μg/L	10,000 μg/L	[228]
Sunitinib	200 μL	Clozapine	Liquid–liquid extraction	ESI+	C18	0.2 μg/L	500 μg/L	[229]
Tamoxifen	50 μL	d5-Tamoxifen	Protein ppt	ESI+	Synergi hydro	5.25 μg/L	1051 μg/L	[202]
Tamoxifen	50 μL	d5-Tamoxifen	Protein ppt	ESI+	C18	0.25 μg/L	1000 μg/L	[203]
Tegafur	100 μL	Strychnine	Protein ppt	ESI+	C18	20 μg/L	5000 μg/L	[196]
Tegafur and 5-fluorouracil	500 μL	5-Chlorouracil	Liquid–liquid extraction	ESI−	C18	25 μg/L (T) 5 μg/L (5FU)	25,000 μg/L (T) 500 μg/L (5FU)	[195]
Thalidomide	Not stated	Thalidomide analogue	SPE	APCI−	C18	2 μg/L	250 μg/L	[198]
Cyclophosphamide and thiotepa	100 μL	Hexamethyl-phosphoramide	Protein ppt	ESI+	C18	200 μg/L (C) 5 μg/L (T)	40,000 μg/L (C) 2500 μg/L (T)	[194]
All-trans retinoic acid	500 μL	Acitretin	SPE	PBI−	C18	0.05 μg/L	50 μg/L	[230]
Vincristine	200 μL	Vinblastine	SPE	ESI+	C8	0.25 μg/L	50 μg/L	[231]
Vincristine and actinomycin D	30 μL	Vinorelbine	Protein ppt	ESI+	C18	0.25 μg/L (V) 0.5 μg/L (A)	100 μg/L (V) 250 μg/L (A)	[232]
Vincristine	500 μL	Vinblastine	Liquid–liquid extraction	ESI+	C18	0.012 μg/L	24 μg/L	[233]
Vincristine	100 μL	Vinblastine	Protein ppt and SPE	APCI+	Luna Phenyl-hexyl	0.1 μg/L	500 g/L	[234]
Vincristine and actinomycin-D	500 μL	Vinorelbine (V) 7-Amino-actinomycin-D (A)	SPE	TIS+	C18	0.5 μg/L	25 μg/L	[235]
Vincristine and actinomycin-D	500 μL	Vinblastine (V) 7-Aminoactinomycin-D (A)	SPE	ESI+	C8	0.5 μg/L	100 μg/L	[236]
Vincristine and vinblastine	1500 μL (VC)2000 μL (VB)	Vinorelbine	Liquid–liquid extraction	APCI+	C18	0.3 μg/L (VC) 0.51 μg/L (VB)	3.95 μg/L (VC) 4 μg/L (VB)	[237]

Table 7
Details of methods for drugs affecting the cardiovascular system.

Drug	Sample volume	Internal standard	Sample preparation	Ionisation mode	Column	LLOQ	Linearity	Reference
Amiodarone	100 μL	d4-Amiodarone	SPE	ESI+	Hydro-RP	0.0075 mg/L	40 mg/L	[244]
Amiodarone	Not stated	AM-25	LLE	ESI+	C18	0.1 mg/L	5 mg/L	[245]
Digoxin	200 μL	Digitoxin	SPE	ESI+	C18	0.02 μg/L	5 μg/L	[241]
Digoxin (dig), digitoxin (dit), methyldigoxin (mdig) and deslanoside (des)	1 mL	Digoxigenin	SPE	ESI+	PIPA Am hydrogel modified thermoresponsive column	0.2 μg/L (dig, mdig, des) 0.3 μg/L (dit)	25 μg/L (dig, mdig, des) 50 μg/L (dit)	[242]
Digoxin	200 μL	d3-Digoxin	LLE	ESI+	C18	0.1 μg/L	10 μg/L	[238]
Digoxin, digitoxin, lanatoside C (lan), acetyldigitoxin (adig)	4 mL	Oleandrin	LLE	ESI+	C18	0.25 μg/L (dig) 0.2 μg/L (dit) 0.6 μg/L (lan) 0.15 μg/L (adig)	100 μg/L	[239]
Digoxin	80 μL	Digitoxin	LLE	ESI+	C18	0.5 μg/L	100 μg/L	[240]
Rivaroxaban	200 μL	Structural analogue	PP	ESI+	C18	0.5 μg/L	500 μg/L	[246]

which is an advantage when quantifying digoxin as there are many substances which cause positive interference in the immunoassays, including digoxin-like substances, digoxin metabolites and spironolactone [243]. However, digoxin analysis is often required on an urgent basis and it may be difficult to provide a result within the required turnaround time using LC–MS/MS analysis, and automated immunoassays remain the method of choice for many laboratories.

The anti-arrhythmic drug amiodarone has been considered as a candidate for therapeutic drug monitoring as adverse effects include pulmonary toxicity, thyroid dysfunction, phototoxicity and tremor, and it was thought that these could be related to circulating levels of digoxin. LC–MS/MS methods have been published using SPE [244] and LLE [245]. Use of d4-amiodarone as an internal standard with solid phase extraction and a hydro-RP analytical column seems to give the best sensitivity and linearity [244]. However, there is no convincing evidence to show that the development of adverse effects can be related directly to circulating amiodarone levels [245], and TDM of amiodarone is not routinely carried out.

Another class of drugs for which TDM may be useful in certain circumstances is the anti-thrombolytic drugs, used in the treatment of stroke and myocardial infarction. Routine TDM is not required, but may be helpful clinically if re-thrombosis occurs during anti-thrombolytic therapy to ascertain if the cause is atypical drug metabolism or variable compliance with therapy. Rivaroxaban, a new generation anti-thrombolytic can be quantified by LC–MS/MS using a C18 column and simple protein precipitation as sample preparation [246]. This is likely to be a rarely required assay, ideally suited to batch analysis by LC–MS/MS (Table 7).

10. Conclusion

LC–MS/MS is a very useful tool for therapeutic drug monitoring, especially if problems such as metabolite interference or lack of a suitable chromophore make alternative methods unsuitable. Although LC–MS/MS analysis does require manual sample preparation, more automated methods are becoming available, such as online SPE instrumentation and pipetting robots which can make liquid–liquid extraction very simple and quick to perform, enabling analysis of large numbers of samples with minimal hands-on preparation. Multiplexed assays which enable simultaneous analysis of commonly co-prescribed drugs such as antiretrovirals or immunosuppressants can be useful as they minimise the need for multiple separate sample preparations, enabling a full panel of results to be produced more quickly, often using less sample volume than separate analyses.

Full validation of methods is paramount to ensure precise and accurate results can be produced. The sensitivity and linearity of methods must be determined and assays developed to make certain that the analytical range of the assay is sufficient to cover the expected plasma concentrations. Interference studies should be carried out to exclude the possibility of interference from drug metabolites or co-prescribed drugs. Sample preparation can be optimised to minimise matrix effects in assays, with liquid–liquid and solid phase extraction proving popular in TDM. Internal standards also go a long way towards minimising matrix problems such as ion suppression. Isotopic internal standards are superior to structural analogues as they behave more like the drug of interest in the assay and are therefore more able to compensate for any differences in ionisation or extraction efficiency. Isotopic internal standards are not always available, however, and if a structural analogue is chosen, extra care should be taken to ensure the analyte of interest elutes well away from any areas of ion suppression and that matrix effects are fully investigated during validation.

The use of matrices other than blood or serum for TDM is becoming more popular. Saliva analysis may offer the opportunity to

quantify free drug levels, which may be important for highly protein bound drugs such as phenytoin, as only the free fraction of the drug is physiologically active. Measurement of the free drug fraction in blood is notoriously difficult and time-consuming as the equilibrium between free and bound drug is easily disturbed during sample preparation. Protein bound drugs cannot enter saliva, so saliva analysis may prove to be a simpler way of quantifying the amount of active drug in the body. Hair analysis has also been proposed as a method of monitoring longer-term patient compliance with prescribed drug therapy. Hair is not yet established as a suitable matrix for TDM, but it could be a convenient, non-invasive way of monitoring patients in the future, once the correlation between hair and plasma levels of drugs has been established and simple sample preparation methods have been developed. The use of dried blood spots for TDM is also becoming more popular, as DBS analysis enables patients to take the samples themselves at home and post them to the laboratory. This can be far more convenient for the patient and even enable AUC analysis for accurate drug monitoring without the patient needing to spend the day in a hospital.

Although methods are available for the quantification of many types of drugs, TDM is not always necessary or appropriate. Therapeutic drug monitoring is routinely reserved for those drugs whose effect cannot be monitored clinically, which have a narrow therapeutic index and a clearly defined relationship between plasma concentration and clinical effect. There are, however, some situations in which TDM may be appropriate, even when the drug in question does not meet the above criteria, for instance in polypharmacy, where drug interactions may affect drug metabolism and excretion, in suspected toxicity, and in cases where poor compliance or an idiosyncratic reaction to a drug is suspected. TDM may be carried out primarily to ensure compliance with treatment in some situations, such as for patients on a drug rehabilitation programme being treated with methadone, or patients treated with opioid analgesics, to prevent overdosage and help stop abuse or diversion of such drugs. In some cases, such as that of thiopurine drugs, it is known that the enzyme responsible for drug metabolism exists in many different forms with different levels of activity, so it is important to measure the phenotype or genotype of the enzyme prior to exposing the patient to the drug, to avoid severe toxicity, rather than measure plasma concentrations after exposure, when the toxicity may have already occurred. As more becomes known about pharmacokinetics and genetic polymorphisms responsible to variabilities in drug metabolism, this may become a more common approach to TDM, with testing carried out prior to drug prescription, enabling dosage to be individualised and the effects of a particular dose to be more accurately predicted.

In conclusion, LC–MS/MS has enabled TDM to be performed on many drugs that could otherwise not be quantified, and in multiple matrices such as blood, hair and saliva. LC–MS/MS is not without its pitfalls, but careful choice of sample preparation method and internal standard, and full validation of assays will avoid the majority of problems and enable accurate drug quantification.

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