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Review

Separation methods for nucleoside analogues used for treatment of HIV-1 infection

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Abstract

Clinicians design antiretroviral therapy to prevent HIV-1 replication and resistance, and researchers study antiretroviral concentrations to understand the pharmacokinetics of these drugs. Because drug efficacy and toxicity varies widely between patients receiving the same antiretroviral therapy, there is interest in monitoring individual patient concentrations of antiretroviral drugs. Good science and effective medical care demand inexpensive validated methods with high throughput that are capable of simultaneously analyzing multiple antiretroviral drugs in various matrices. Currently, protease inhibitors, non-nucleoside reverse transcriptase inhibitors, and nucleoside reverse transcriptase inhibitors are used to treat HIV-1 infection. This review summarizes published methods for the quantitation of nucleoside reverse transcriptase inhibitors and their metabolites in different matrices using immunoassays, ultraviolet absorption, and mass spectrometry.

Keywords: Reviews; Nucleoside reverse transcriptase inhibitors

Contents

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1. Introduction

Nucleoside reverse transcriptase inhibitors (NRTIs) comprise the first class of compounds developed to treat the acquired immunodeficiency syndrome (AIDS) caused by the human immunodeficiency virus (HIV-1). NRTIs are synthetic 2′,3′-dideoxynucleoside analogs of naturally occurring nucleosides. The six NRTIs, approved for use against HIV-1, infection are: zidovudine (ZDV), didanosine (ddI), zalcitabine (ddC), stavudine (d4T), lamivudine (3TC), and abacavir (ABC) (Fig. 1). A typical therapeutic regimen contains one or more NRTI in combination with an HIV-1 protease inhibitor, a non-nucleoside inhibitor of HIV-1 reverse transcriptase, or both [1].

NRTIs are inactive prodrugs, which must be phosphorylated by intracellular enzymes to their respective dideoxynucleoside-5′-triphosphate for activity. NRTIs have two modes of action. First, triphosphorylated NRTIs (NRTI-TP) competitively inhibit viral reverse transcriptase. Second, because NRTIs lack the 3′-hydroxyl needed for 5′→3′ phosphodiester DNA propagation, their incorporation into viral DNA prevents further viral replication and blocks the viral life cycle [2].

The goal of NRTI therapy is prevention of viral replication with minimal adverse effects. Since the pharmacokinetics of NRTIs vary between individuals, monitoring of patient plasma drug concentrations is becoming common. However, plasma concentrations may not reflect drug concentrations in other physiological compartments that harbor HIV-1 [3,4]. Therefore, it may be important to monitor NRTIs in all pathologically relevant compartments, including the central nervous system, the genital tract, and breast milk. Patients may also benefit from monitoring NRTI concentrations in urine, a major route of NRTI elimination. There is evidence that extracellular concentrations of NRTIs do not correlate with virological response or reflect intracellular concentrations of NRTI-TPs. Instead, intracellular concentrations of NRTI-TP may correlate better with virological response and clinical outcome. Consequently, monitoring of NRTI-TPs may be more appropriate. Finally, a better understanding of intracellular AMT (3′-amino-3′-deoxythymidine), a cytotoxic metabolite of ZDV, concentrations may give a better insight into ZDV toxicity [1]. Clearly, researchers need sensitive high throughput methods specialized to study NRTIs and their metabolites in various matrices.

2. Choosing the analytical method

When choosing a method, factors to consider include: time needed to complete analysis, necessary

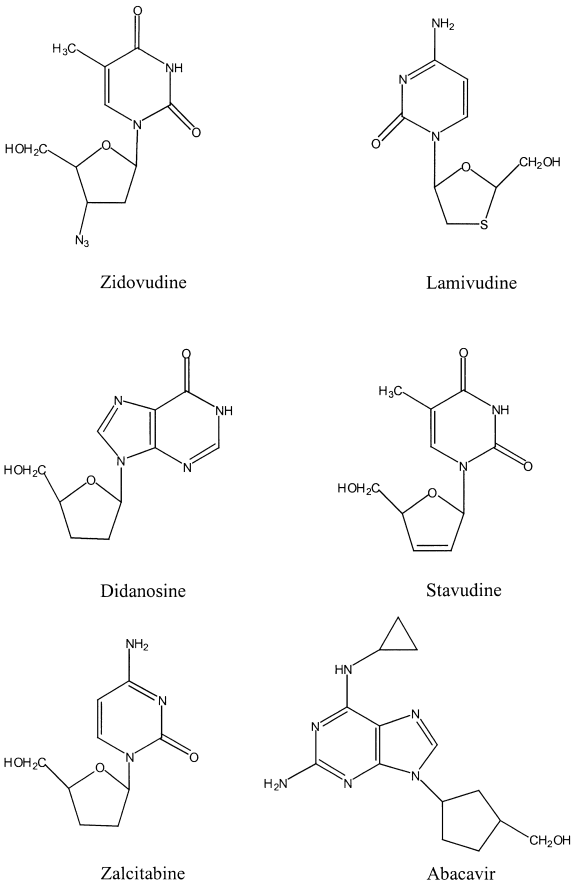

Fig. 1. Chemical structures of NRTIs.

equipment, sample size required, target analyte, and matrix of interest. For example, methods that require a large sample size may be inappropriate for some populations or matrices. The target analyte may be NRTI prodrug, NRTI-TP, other NRTI metabolite, or a combination of multiple NRTIs and NRTI metabolites. Therefore, a method that simultaneously quantifies multiple NRTIs with multiple metabolites may be more efficient and cost effective. Finally, methods designed to quantify NRTIs in plasma may not be useful when the sample is composed of urine, intracellular components, or other matrix.

Generally, the most reliable methods are those which have been validated. Method validation ensures the specificity, accuracy, and precision of an analytical assay and assesses the stability of the analyte during storage and handling. Accuracy describes the agreement between measured and nominal concentrations. Precision is the agreement among replicate measurements. Within-day (intra-assay) precision is the variation among multiple replicates in a single assay. Between-day (inter-assay) precision is the variation across multiple days, or runs. Other parameters of interest include recovery from sample processing and the calibration range of the method.

Because scientists analyze hundreds and sometimes thousands of samples during clinical trails, analytical methods must be rapid. In general, most HPLC–UV (high-performance liquid chromatography coupled with ultraviolet absorbance detection) methods and all intracellular targets require extraction from sample matrix. Extractions, including liquid–liquid extraction (LLE), solid-phase extraction (SPE), and column switching, increase sample preparation time. The faster sample preparation techniques of protein precipitation and ultrafiltration are relatively dirty, limiting the use of UV (ultraviolet absorption) analysis. Immunoassays and HPLC–MS (high-performance liquid chromatography coupled with mass spectrometric detection), because of their highly specific and sensitive nature, reduce the need for lengthy sample preparation. However, the cost of a mass spectrometer can be prohibitive and immunoassays are not designed to quantify multiple NRTIs simultaneously.

When complicated sample preparation is required, an internal standard can be useful. Internal standards compensate for analyte loss during sample preparation [5]. UV detection methods use surrogate compounds as internal standards. However, chromatographic separation between analyte and internal standard generally requires additional chromatographic run time. Stable isotopically labeled analyte can be used as the internal standard during HPLC–MS–MS (high-performance liquid chromatography coupled with tandem mass spectrometric detectors), eliminating the need for additional chromatography [6,7]. By contrast, immunoassays typically do not employ internal standards [8].

Finally, the amount and type of waste generated by different analytical methods is of importance. Most methods involving HPLC–UV use a flow of 1 ml/min or faster and generally take over 30 min. By contrast, most HPLC–MS methods perform better at

a flow less than 0.5 ml/min and generally last less than 10 min. The amount of hazardous waste generated by MS (mass spectrometric) methods, therefore, is considerable less than UV methods. The use of radioisotopes during radioimmunoassay (RIA) is also worthy of consideration. Following RIA, researchers must dispose of radioactive clinical waste.

3. Methods for quantifying NRTIs

3.1. Immunoassay as the detection step

3.1.1. Radioimmunoassay involving methods

Radioimmunoassay uses radioisotopic tracers (radiolabeled analyte) to detect and quantify the analyte of interest. The tracer competes with analyte for detection by analyte specific antiserum. A secondary antibody precipitates the tracer–antiserum and analyte–antiserum complexes. The precipitate is resuspended in fresh solvent and its radioactivity is determined. There is an inverse relationship between amount of radioactivity and concentration of analyte [8].

3.1.1.1. Zidovudine. In 1989, Quinn et al. [9] authored the first paper for quantifying ZDV in human plasma by RIA. In short, this method required no extraction and only 100 μl sample. As with most RIAs, the sample, ZDV antiserum, and tracer, [^{3}H]ZDV were added to a centrifuge tube. However, at the time of publication, neither ZDV-antiserum nor the immunogen needed to immunize rabbits against ZDV was commercially available. Following centrifugation, the precipitate was washed and re-suspended in acid. Finally, scintillation counting fluid was added and the radioactivity was determined by a scintillation counter. Calibration standards (3–800 n*M*) were prepared in human plasma. Over the range of calibration concentrations, intra-assay and inter-assay coefficients of variation were less than 10 and 30%, respectively. Accuracy was greater than 80%. The authors found no cross-reactivity with common medicines or with ZDV-G, the glucuronidated metabolite of ZDV. The method was used to measure ZDV in rats dosed with ZDV. These results, according to the authors, correlated well with simultaneous measurements by HPLC–UV. This method has been used to measure ZDV in plasma, urine, and tissue samples collected from patients receiving ZDV therapy.

A year later, Tadepalli et al. [10] designed a method that cited a commercially available RIA kit (Incstar Corporation) for the quantitation of ZDV and ZDV-G in serum and urine. While ZDV quantitation was direct, ZDV-G quantitation required an indirect approach. Briefly, the authors used the RIA kit to measure ZDV before and after hydrolysis with β-glucuronidase and subtracted the pre-hydrolysis ZDV concentration from post-hydrolysis ZDV concentration. The difference represented the concentration of ZDV-G. ZDV quantitation proceeded as follows. First, the sample (100 μl of serum or urine) was mixed and incubated with rabbit ZDV antiserum and tracer ([^{125}I]ZDV). Goat anti-rabbit IgG (Immunoglobulin G) was added to precipitate the rabbit antisera. Following centrifugation, the supernatant was discarded and the radiation in the pellet was measured with a gamma counter. Neither ZDV-G, nor β-glucuronidase, nor any of the other compounds tested cross-reacted in this assay. Recovery of ZDV and ZDV-G from urine and serum was greater than 95%. ZDV calibration standards ranged from 66.8 to 534.4 μg/l in serum and 334 to 2670 μg/l in urine. ZDV-G calibration standards ranged from 221.7 to 1770 and 1660 to 13300 μg/l in plasma and urine, respectively. The accuracy of ZDV and ZDV-G analyses was greater than 85% in both matrices. Intra-assay and inter-assay coefficients of variation were less than 12% for both analytes in either matrix. Use of [^{125}I]ZDV instead of [^{3}H]ZDV allowed for a shorter incubation and more efficient counting as compared to the previous method by Quinn et al. [9]. However, radioactive risk was increased with the more penetrating ^{125}I. The authors have used this procedure to measure ZDV and ZDV-G in the serum and urine of patients receiving ZDV therapy.

Stretcher et al. [11] used the ZDV RIA kit to measure total intracellular ZDV-phosphates in cells cultured with ZDV. Again, an indirect approach was used. Total ZDV-phosphate (ZDV-MP, ZDV-DP, plus ZDV-TP) represented the difference between two aliquots of cells cultured with ZDV. One aliquot underwent hydrolysis with alkaline phosphatase and the other forewent hydrolysis. The difference in measured ZDV concentration was attributed to the

concentration of ZDV-phosphates. Like β-glucuronidase, Stretcher et al. [11] demonstrated that alkaline phosphatase did not interfere with RIA analysis. The authors did not report the accuracy, precision, or sample size required. This method did not separate the different ZDV-phosphates. Instead, it reported a total intracellular ZDV-phosphate concentration.

The method developed by Kuster et al. [12] did measure intracellular ZDV-TP. The procedure used HPLC (SAX analytical column, linear gradient from 12 to 120 m*M* KH_2PO_4 at a flow of 2 ml/min) to separate ZDV-phosphates into separate fractions. The ZDV-TP fraction was hydrolyzed with alkaline phosphatase, cleaned with solid-phase extraction (SPE) (C_{18} cartridges, methanol elution), dried, re-suspended, and analyzed with the commercial ZDV RIA kit. The method required 20 ml whole blood. Recovery of ZDV-TP from HPLC–SPE–RIA, as determined by spiking known amounts of ZDV-TP into cell extracts and processing according to protocol, was 100–120%. While this method appeared to be relatively easy, there was no report regarding its accuracy or precision. Further, Kuster chose to prepare the ZDV-TP standards (1.1–272 ng/200 μl) in distilled water, which may not be an appropriate matrix for standards used to calibrate cell extracts. Because the inorganic phosphates of the HPLC mobile phase inhibited alkaline phosphatase, the three fractions must incubate with the enzyme for 24 h. Other authors have shown different mobile phases eliminate the need for such a long incubation time. ZDV-MP and ZDV-DP standards were not created, yet the method was used clinically to measure intracellular concentrations of all three ZDV-phosphates. The authors also admitted the assay was not sensitive enough to detect intracellular ZDV-DP or ZDV-TP 4-h post-dose.

Slusher et al. [13] also published an HPLC–SPE–RIA method for the measurement of intracellular ZDV-phosphates. Like Kuster et al., Slusher et al. used gradient HPLC, with increasing concentrations of potassium phosphate buffer, to elute the different ZDV-phosphates from a SAX analytical column into separate fractions. In this case, the author used acid phosphatase to digest the ZDV-phosphates. The digested fractions were passed through C_{18} SPE cartridges, eluted with methanol, dried, and reconstituted. In addition, the collected fractions underwent an additional cleanup by a second HPLC system (C_{18} analytical column, isocratic methanol–water, 36:64, v/v, at 1 ml/min). The ZDV fraction of the second HPLC system was dried, reconstituted in RIA buffer, and analyzed with the commercial ZDV RIA kit. It is important to note that Slusher chose to prepare aqueous ZDV standards, which were not exposed to the entire HPLC–SPE–HPLC–RIA protocol. Instead, the aqueous standards were introduced only at the final, RIA, step. Preparation of individual ZDV-phosphate standards in cell extract and introduction of standards at the start of the protocol rather than at the RIA step would have better represented the handling of clinical samples. The author compared [^{3}H]ZDV-phosphate concentrations radioisotopically and by HPLC–SPE–HPLC–RIA. Based upon the results, Slusher determined the method was validated. Like Kuster et al., Slusher et al. did not mention the accuracy or precision of his assay. Additionally, Slusher et al. never calculated the recovery of ZDV-phosphates from cell extracts using HPLC–SPE–HPLC–RIA.

Peter et al. [14] also authored a method using the commercial ZDV RIA kit for the quantitation of ZDV-phosphates. Essentially the method contained three steps: HPLC, SPE, and RIA. Fractions corresponding to ZDV-phosphates were collected from the HPLC system (C_{18} analytical column, phosphate buffer–tetrabutyl ammonium phosphate–acetonitrile, 957.3:7.7:3.5, v/v/v, at 1 ml/min). Tetrabutylammonium phosphate was used, despite evidence that it elevated background noise. The fractions were collected, dried, reconstituted in phosphate buffer, and incubated with alkaline phosphatase for 20 h. SPE, with methanol elution, was used to clean up the hydrolyzed fractions. The SPE eluent was dried, reconstituted in RIA buffer, and subjected to the commercial ZDV RIA kit. Analysis required 30 ml whole blood. Calibration standards (0.21–10.5 ng/ml) consisted of ZDV in HPLC mobile phase, not individual ZDV-phosphates in cell extract. Thus, the reported accuracy of greater than 85% and inter-assay and intra-assay coefficients of variation less than 9% may not reflect ZDV-phosphate analysis. ZDV-phosphate standards in cell extract and introduction of standards at the start of the protocol rather than at the RIA step would have better represented

the handling of clinical samples. This method has been used to measure intracellular ZDV-phosphates in HIV-1 positive patients receiving ZDV.

Robbins et al. [15] used SAX SPE cartridges to separate ZDV, ZDV-MP, ZDV-DP, and ZDV-TP. The void fraction contained ZDV. Washing the cartridges with 6 ml 74.5 m*M* KCl eluted ZDV-MP, with 11 ml KCl eluted ZDV-DP, and with 3.3 ml 1 *M* KCl eluted ZDV-TP. Acid phosphatase was added to the ZDV-phosphate fractions. Digested fractions were cleaned with C_{18} SPE cartridges (eluted with 5% methanol), and subjected to the commercial ZDV RIA kit. By comparing cell extracts spiked with radiolabeled ZDV-phosphates both radioisotopically and by this method, recoveries of ZDV-MP and ZDV-TP were 90%. Because phosphate buffers were avoided, the hydrolysis step takes only 30 min rather than 20+ h required by other methods. Concentrations of ZDV-TP and ZDV-MP measured in cells incubated with ZDV determined both by HPLC and by this method were within 16% of each other. Accuracy and reproducibility were assessed using spiked PMBC extracts with known concentrations of ZDV-MP (range 0.7–12 pmol/10^6 cells) and ZDV-TP (range 0.05–0.502 pmol/10^6 cells). Bias and inter-assay coefficient of variation of ZDV-MP analysis were less than 20%. Unfortunately, bias of ZDV-TP reached as high as 32% at 0.05 pmol/10^6 cells. Perhaps use of individual ZDV-phosphate standards prepared in cell extracts, rather than aqueous ZDV standards, would have been a better choice. This method has been used to study intracellular concentrations of ZDV-MP and ZDV-TP from cells collected from patients receiving ZDV therapy.

3.1.1.2. Lamivudine. Robbins et al. [16] also published a RIA method for the quantitation of intracellular 3TC-TP. Because 3TC antiserum was not available commercially, a 3TC immunogen had to be prepared and used to produce rabbit 3TC antibodies. The tracer, [^{3}H]3TC, was available commercially from Moravek Biochemical. The method was similar to those described above for the quantitation of intracellular ZDV-TP. Briefly, cell extract from 16 ml of whole blood was passed through C_{18} SPE cartridges. 3TC, 3TC-MP, and 3TC-DP were eluted together by 8 ml of 9.14 m*M* KCl. 3TC-TP was eluted with 5 ml of 300 m*M* KCl. The 3TC-TP fraction was incubated with 1.5 U/ml acid phosphatase for 60 min and analyzed by RIA. During RIA, 3TC antiserum and [^{3}H]3TC were added, followed by goat anti-rabbit IgG. This mixture was centrifuged, the supernatant discarded, and the pellet re-suspended in HCl. Finally, a scintillation counter determined the amount of radioactivity remaining. The authors found no significant cross-reactivity with cytidine analogs or with other antiretrovirals. Standards (0.097–12.5 ng/ml) were created using 3TC in water. As stated above, standards composed of 3TC-TP in cell extract may better represent patient samples. Over the range of calibration standards, intra-assay and inter-assay coefficients were between 1.5–15% and 12–21%. Accuracy was greater than 95%. Samples analyzed by this method were within 15% of an HPLC method, giving further evidence of the observed accuracy and precision. Although not a simultaneous assay, the author combined this method with his earlier ZDV-TP RIA. He suggested using four million cells (4 ml whole blood) to measure 3TC-TP and 16 million cells (16 ml whole blood) for ZDV-TP analysis. However, this method was validated using a 16 ml sample size. It is likely that a smaller sample size will make quantitation at the lower end of the calibration curve difficult. This combination has been used to measure ZDV-TP and 3TC-TP in patient samples.

3.1.1.3. Zalcitabine. Roberts et al. [17] published an RIA method for measuring ddC in 2 ml plasma. The RIA tracer, [^{3}H]ddC, and rabbit ddC antiserum were available from Sigma. Before RIA, samples were cleaned using SCX SPE cartridges. The RIA followed a predictable protocol. Standards of ddC (0.4–25 μg/l) were prepared in human plasma. The antiserum showed no cross-reactivity of metabolites of ddC or to several other drugs. Over the range of calibration standards, accuracy, inter-assay precision, and intra-assay precision were greater than 90, 83, and 80%; respectively. Recovery of [^{3}H]ddC from SCX SPE was 89%. Recovery of other NRTIs was very poor ([^{3}H]ZDV and [^{3}H]ddI both less than 5%). However, authors speculated that other cytidine analogs, including ddC-phosphates, would be extracted from plasma by SCX SPE. Data from one subject demonstrated that the method was sensitive

enough to quantitate trough concentrations of ddC. Thus, this method may be useful for performing detailed pharmacokinetic studies.

Kastrissios et al. [18] developed a C_{18} SPE–RIA for ddC requiring only 500 μl sample. Extraction efficiency was 89%. Like Roberts et al., ddC standards (0.2–20 μg/l) were made in blank plasma and RIA reagents were obtained from Sigma [17]. Again, no interference from drug-free plasma or from common HIV-1 drugs was found. The error of this method ranged from 5 to 13% and within-day imprecision reached as high as 20%. Between-day imprecision was not reported. Like Roberts et al., Kastrissios et al. have used this method in the clinical setting.

3.1.1.4. Stavudine. Zhou et al. [19] developed a RIA method for measuring d4T in human plasma. While the tracer, [^{3}H]d4T, was available commercially from Moravek Biochemical, the d4T immunogen had to be prepared and used for production of rabbit d4T antibodies. The d4T calibration standards (5–500 ng/ml) were prepared in spiked human plasma. Intra-assay coefficients of variation were less than 3.5%. However, inter-assay coefficients of variation reached 17%. Error was less than 21%. The method followed typical RIA protocol, as outlined above. This method has been used to measure d4T in clinical samples.

3.1.1.5. Didanosine. DeRemer et al. [20] published a RIA method for the quantitation of ddI in human plasma. The tracer, [2',3′-3H]ddI, was available from Morvavek Biochemical and rabbit ddI antiserum was available from Sigma. Again, the method followed the typical RIA scheme, with detection of the tracer via scintillation counting. Sample size was only 100 μl plasma. Accuracy at 100 ng/ml was only 48%. At 50 ng/ml the accuracy rose to a much more reasonable 92%. Inter- and intra-assay coefficient of variations were less than 15% throughout the calibration range (0.4–400 ng/ml). Because the authors prepared their standards in buffer, these data may not reflect the accuracy or precision of plasma analysis. Stability testing demonstrated that ddI was stable at room temperature and over multiple freeze–thaw cycles. Specificity of the assay was tested against samples containing drugs commonly used in HIV-1 treatment, and no significant cross-reactivity was found. The authors claimed this method could detect ddI in cerebral spinal fluid (CSF) and urine. However, they also admitted false positives appeared during analysis of blank urine. This assay has been used in the HIV-1 infected patient population.

3.1.1.6. Abacavir. There has been no published RIA method quantifying ABC.

3.1.2. Enzyme-linked immunosorbent assay involving methods

Unlike RIA, enzyme-linked immunosorbent assay (ELISA) uses no radioisotopes. Instead, the UV absorbance or optical density of the sample is monitored. The intensity of the sample color is determined by the amount of the product produced from an enzyme catalyzed reaction. The enzyme is conjugated to either the primary antibody, the secondary antibody, or to the analyte itself. In any case, only the enzyme immobilized to the microtiter plate can react with substrate to produce a colored product. Thus, color intensity is directly related to amount of analyte in the sample [8].

3.1.2.1. Zidovudine. Tadepalli and Quinn [21] described an ELISA for the quantification of ZDV in 100-μl samples of human serum. However, because the primary antibody reacts with G-ZDV, a liquid–liquid extraction (LLE) of ZDV from plasma is required. Briefly, acetonitrile and saturated NaCl were added to the sample. The organic layer was collected, dried, and reconstituted in PBS. Extraction efficiency was reported to be 95%. Sample, mouse ZDV antiserum, and anti-mouse IgG-alkaline phosphatase were incubated together in microtiter plate wells coated with a ZDV-rabbit serum albumin conjugate. Following this incubation, unbound materials were washed from the plate and the substrate, *p*-nitrophenylphosphate in diethanolamine, was added to the wells. Addition of substrate produced a color change detectable by a plate reader capable of determining changes in optical density at 405 nm. The calibration range, 125–4000 n*M* in human serum, had an intra-assay precision better than 92% and an inter-assay precision better than 83%. The

standards were consistently within 12% of their nominal concentration. Neither the coating antigen nor the primary antibody was commercially available. No clinical data was provided.

3.1.2.2. Stavudine. Ferrua et al. [22] published an ELISA method for measuring d4T in serum. Sample analysis involved ultrafiltration followed by ELISA. Recovery of d4T from serum by ultrafiltration was 81%. The ELISA protocol began with microtiter plates coated with anti-rabbit IgG. Sample (500 μl), d4T–horseradish peroxidase conjugate, and rabbit anti-d4T were incubated in the plate wells. Next, unbound constituents were washed from the plate and a peroxide substrate (OPD/H_2O_2, *o*-phenylene dihydrochloride in a hydrogen peroxide solution) was added to the wells. Finally, the concentration of product produced from the OPD/peroxidase reaction was determined using an automatic plate reader capable of measuring absorbance at 492 nm. Neither the d4T–horseradish peroxidase conjugate nor rabbit d4T antiserum was available commercially. Standards (0–10 000 ng/ml) were made in ELISA diluent, and not plasma. Inter- and intra-assay coefficients of variation were between 6 and 14%. Specificity was analyzed by studying cross-reactivity of natural and synthetic nucleosides with d4T antiserum, and no significant cross-reactivity was found. Ferrua et al. suggested that this method, coupled to HPLC separation and hydrolysis of d4T-phosphates, could be used to detect individual intracellular d4T-phosphates. However, the authors reported that this separation technique did not fully separate d4T-phosphates. This assay was used to monitor patient plasma d4T concentrations.

3.1.2.3. Lamivudine, zalcitabine, didanosine, or abacavir. No one has published an ELISA for measuring 3TC, ddC, ddI, or ABC.

3.1.3. Fluorescence polarization immunoassay involving methods

During fluorescence polarization immunoassay (FPIA), drug (analyte) in the sample competes for antibody with fluorescein-labelled tracer [5,8]. The mixture is illuminated with plane polarized light (PPL), causing excitation of fluorescein. Upon relaxation, fluorescein emits PPL, which is detected by a fluorometer. When PPL is used as the excitation source, molecules can only be excited while in proper alignment. Smaller, antibody-free fluorescein-labelled drug rotates faster than larger, antibody-bound fluorescein-labelled drug. Thus, antibody-bound fluorescein-labelled drug is more likely to become excited and emit fluorescent light. Fluorescence (F) can be described as $F = Kc$, where K is an intrinsic factor relating the efficiency of fluorescence and c is the concentration of fluorescent species. Therefore, there is an inverse relationship between unlabeled drug and emission of PPL. In order to use FPIA, a fluorescence detector capable of emitting PPL is needed. Because of the prohibitively high cost of FPIA instrumentation, it is unlikely that this technique will be useful for smaller laboratories or for routine clinical analysis. However, no time-consuming wash or re-suspension steps, availability of system automation, and ease of use some are advantages of FPIA over other immunoassays. Like ELISA, FPIA does not require the use of radioisotopes.

3.1.3.1. Zidovudine. Granich et al. [23] described a competitive FPIA capable of measuring ZDV in 50–200 μl serum. Briefly, samples underwent protein precipitation followed by FPIA. The procedure required fluorescein-labelled ZDV, which was not available commercially. The assay cross-reacted with the ZDV analogs 3′-methylthymidine, 3′,5′-dideoxythymidine, and AzdU, but not with 42 commonly prescribed drugs. Recovery of ZDV in serum, as compared to an aqueous solution, was 93%. Intra-assay precision and inter-assay precision were both greater than 90%. It is important to note, however, that the authors prepared their standards (51–3928 ng/ml) in calf, not human serum. While it was unclear whether this matrix substitution would affect sample analysis, drug-free human serum was easy to obtain and would have better approximated patient serum. ZDV was stable during heat inactivation of HIV-1 (56°C, 30 min). Accuracy of this method was comparable to HPLC and the commercially available ZDV RIA kit, however no error value was stated. The authors suggested that this method might be

capable of determining G-ZDV concentrations, using the indirect approach described earlier.

3.1.3.2. Lamivudine, zalcitabine, stavudine, didanosine, abacavir. Currently, no other FPIA exists in the literature for the measurement of these NRTIs.

3.2. Ultraviolet absorbance as the detection step

Ultraviolet spectrophotometry uses the intrinsic ability of a compound to absorb ultraviolet (UV) light as a means of determining their concentration in solution [5]. Beers law describes absorbance of UV light (A) as $A = abc$, where a is a molecule's inherent ability to absorb light, b is the cell length and c is the concentration of molecule in solution. In general, UV absorbance is directly related to the concentration of analyte in the sample. However, because multiple species absorb UV light, unambiguous identification is nearly impossible. Thus, if multiple UV absorbing compounds co-elute, there will be interference. This can be an advantage, however, as the same piece of equipment can be used to monitor multiple compounds, if their introduction into the UV cell is adequately separated.

3.2.1. Zidovudine

Unadkat et al. [24] published one of the first HPLC–UV methods for analyzing ZDV in 25–500 μl of rat plasma or urine. The commercially available internal standard, *p*-hydroxyphenobarbitol, was added to plasma and urine samples at the start of the method. The initial plasma cleanup entailed LLE, with ethyl acetate–diethylether (50:50, v/v) as the extraction solvent. The organic layer was saved, dried, and reconstituted in HPLC mobile phase. Recovery from LLE was 65%. The reconstituted sample was then injected onto the HPLC system (C_{18} analytical column, phosphate buffer–acetonitrile, 80:20, v/v). Urine samples were simply diluted with the internal standard solution and directly injected onto the HPLC system. Absorbance was monitored at 266 nm. Standards were made in either water or urine, depending on the matrix of study. ZDV in bovine serum albumin, not plasma, was used for calibrating plasma analysis. Between-day variation was 2.9%, as determined by daily differences in the slope of the calibration curve. Within-day variation was less than 6%, and accuracy was greater than 90%. The author used this method to study ZDV in rat plasma and urine following bolus intravenous administration of ZDV.

Hedaya and Sawchuk [25] published a method for determining ZDV concentrations in human plasma and urine. The method called for either 1 ml plasma or 100 μl urine. The author chose β-hydroxypropyltheophylline as the internal standard. Like Unadkat et al. [24], LLE (chloroform–isoproyl alcohol, 95:5, v/v, as extraction solvent) preceded plasma analysis and urine analysis mandated simple dilution with water. Following LLE, the organic layer was saved, dried, and reconstituted in HPLC mobile phase (phosphate buffer–acetonitrile, 91:9, v/v). A C_{18} analytical column eluted at 1.5 ml/min and a detector set to monitor absorbance at 266 nm comprised the HPLC system. Standards were made in either plasma (0.015–2.0 mg/l) or urine (0.3–30 mg/l). Within-day and between-day coefficients of variation were less than 15% for the two matrices. The error for either matrix was less than 10%. Comparison of extracted sample (plasma or urine) with unextracted aqueous solution of ZDV yielded a recovery of almost 70%. The author chose to construct calibration curves using peak height ratios, as opposed to the more appropriate peak area ratios. The author reported no chromatographic interference with 16 over the counter or prescribed drugs. Hedaya has used this method to study ZDV in the plasma and urine of healthy human volunteers.

Good et al. [26] described a method for determining ZDV and ZDV-G concentrations in serum. The internal standard, available only from Glaxo Wellcome (now Glaxo Smith Kline), was the 3′-β-azido isomer of ZDV. The internal standard was added to a 500-μl sample. Samples were introduced to C_{18} SPE cartridges, eluted with methanol, dried, reconstituted in HPLC mobile phase (phosphate buffer–acetonitrile, 85:15, v/v), and introduced onto the HPLC system. The HPLC system consisted of a C_{18} analytical column and the eluent (1 ml/min) was monitored at 267 nm. Elution of ZDV, ZDV-G, and the internal standard was isocratic, but a gradient was used to wash and re-equilibrate the analytical column. Recoveries of ZDV and ZDV-G extracted

from serum were 93% of unextracted aqueous samples. ZDV (0.1–20 μ*M*) and ZDV-G (0.2–40 μ*M*) standards were prepared in serum. However, because ZDV-G was not available commercially, the author first extracted ZDV-G from urine collected from patients receiving ZDV therapy. Good et al. demonstrated ZDV and ZDV-G stability during heat inactivation of HIV-1 (58°C for 1 h). Error of ZDV and ZDV-G analysis was less than 10%. Inter-assay and intra-assay coefficients of variation were both less than 15%, over the ranges of calibration standards. Because of interference with endogenous molecules, the method could not be used for urine analysis. Of the more than 25 over the counter and prescription drugs tested for interference with ZDV or ZDV-G chromatography, only ddC appeared to interfere with analysis of ZDV-G in serum. Thus, this method may not be useful for analyzing plasma samples from patients receiving multiple NRTIs. The author claimed routine use of this method for monitoring plasma and serum ZDV and ZDV-G.

Nadal et al. [27] amended the method described by Good et al. to measure ZDV (0.025–2.5 μg/ml) and ZDV-G (0.05–10.0 μg/ml) in plasma. First, an ion pairing agent, *n*-octylamine, was added to the HPLC mobile phase to increase the retention time of ZDV-G. Second, gradient HPLC was used to shorten analysis time and prevent peak broadening. Third, the author chose a commercially available internal standard, 7-ethyltheophylline. Finally, diode array detection was used to ensure peak purity. However, this method developed by Nadal et al. requires twice as much sample as the method developed by Good et al. [26] (1 ml plasma versus 500 μl plasma). The recoveries, accuracies, and precision measurements of the two assays were comparable.

Ruprecht et al. [28] demonstrated that it was possible to measure ZDV in murine brain, embryonic tissue, milk, and serum. Analysis required methanol precipitation before samples could be introduced to the HPLC system (C_{18} analytical column, mobile phase of ammonium acetate buffer–acetonitrile, 94:6, v/v, at 1 ml/min, monitored at 280 nm). The authors chose *p*-nitrophenol as the internal standard. Recovery, calculated by comparing ZDV extracted from sample with ZDV extracted from aqueous methanol, was 96–120%. Note, recovery of ZDV extracted from milk was compared to ZDV extraction from methanol–water. The author provided no information regarding the accuracy or precision of this method. Like Hedaya and Sawchuk [25], Ruprecht et al. [28] used peak-height ratios, rather than peak-area ratios, to construct calibration curves. A modern integrator could have calculated peak areas, which are less likely to be dependent on peak broadening. This method has been used to study ZDV in pregnant, lactating, and embryonic mice. The author found ZDV in both milk and embryonic tissue.

Burger et al. [29] described a method for analyzing AMT (3′-amino-3′-deoxythymidine), the suspected cytotoxic metabolite of ZDV. Briefly, 500 μl plasma were diluted with phosphate buffer. The diluted samples were added to SCX SPE cartridges and eluted with 2.5% NH_4OH in methanol. The eluent was dried, redissolved in HPLC mobile phase, and injected onto the HPLC system. Chromatographic conditions included a C_8 analytical column and a methanol–ammonium acetate–sodium dioctylsulfosuccinate in water (60:40:4, v/v/v) mobile phase. Flow was 1.0 ml/min, and absorbance was monitored at 265 nm. Recovery from SPE, as determined by the differences in the slope of unextracted samples in aqueous methanol with spiked plasma samples was approximately 80%. Specificity was demonstrated using blank plasma. Further, the retention times of common drugs were different from the retention time of AMT. Burger demonstrated the stability of AMT during heat inactivation of HIV-1 (60°C for 3 h), freeze–thaw cycles, and long-term storage at −30°C. Calibration standards (5–200 ng/ml) were prepared in plasma. Between-day precision, within-day precision, and accuracy were greater than 85%, throughout the calibration range. It was likely the accuracy and precision could have been even better by employing an internal standard. This method has been used to measure AMT in patient plasma. Clearly, it would also be interesting to measure AMT concentrations in bone marrow cells, where the most deleterious effects of AMT have been recorded.

3.2.2. Lamivudine

Harker et al. [30] developed an HPLC–UV assay for measuring 3TC in serum. The method begins with SPE (methanol–triethylamine, 9:1, v/v, eluent) cleanup followed by injection onto the HPLC system. The HPLC mobile phase consisted of 8%

methanol, 1% acetonitrile, 0.1% acetic acid in 0.1 *M* ammonium acetate made up with distilled water and was pumped at a flow of 1 ml/min. The assay was monitored at 270 nm. With a 1-ml sample, the method was validated from 10 to 5000 ng/ml. By comparing the calibration line of heated and unheated standards, Harker et al. demonstrated the stability of 3TC during heat inactivation of HV (60°C for 3 h). Glaxo Wellcome provided the internal standard, carbovir. Standards were prepared in spiked human serum. Although the intra-assay coefficient of variation at 10 ng/ml was 17.4%, the assay was more robust at higher concentrations. Accuracy, intra-assay precision, inter-assay precision were all greater than 85% at concentrations between greater than 49 ng/ml. Harker found no chromatographic interference by blank serum, by the sulfoxide metabolite of 3TC, or by other nucleoside analogs. This method has been automated and used for human studies. Because ABC is metabolized to carbovir-TP, this method may not be appropriate for use with subjects receiving 3TC and ABC combination therapy.

Morris and Selinger [31] incorporated a column switching scheme into his procedure for measuring 3TC in urine. 3TC calibration standards (0.5–500 μg/ml) were prepared in urine. Morris and Selinger [31] noted less than 5% analyte loss during heat inactivation of HIV-1 (58°C for 5 h), storage at room temperature for 4 days, and storage at −30°C for 24 days. Specificity was studied in drug-free urine and by spiking other compounds, including nucleosides, into urine. No chromatographic interference was detected. Accuracy and intra-assay precision of samples containing 1.50–375 ng/ml 3TC were both greater than 85%. Morris and Selinger [31] did not comment on the inter-assay precision or suggest an internal standard. Because column switching affords minimal sample handling, there was less risk of exposure to HIV-1-infected samples. This method has been used to study 3TC in the urine of HIV-1 infected patients.

Zhou and Sommadossi [32] used trichloroacetic acid protein precipitation as sample cleanup for 3TC analysis in human serum. The supernatant was injected directly onto the HPLC system (C_{18} analytical column, phosphate buffer–methanol, 88.3:11.7, v/v, 1 ml/min, 280 nm). Then, each sample analysis was followed by a 5-min wash and a 10-min column re-equilibration. Students *t*-test found no significant difference between fresh samples and samples subjected to various conditions, including heat inactivation of HIV-1 (58°C for 4 h), freeze–thaw cycles, and room temperature for 24 h. Specificity of the method was confirmed by analyzing, without interference, serum from patients receiving other HIV-1 drugs serum spiked with NRTIs and NRTI metabolites. Standards (20–10 000 ng/ml) were prepared in human serum. Accuracy, inter-assay precision and intra-assay precision were greater than 85%. Of note, this method required only a 100-μl sample, less than the method developed by Harker et al. [30]. Because no extraction was employed, the authors assumed recovery should have been 100%, and chose not to use an internal standard. When extracted serum samples were compared to unextracted aqueous samples, recovery was 85%. An internal standard might have been able to account for this loss. The author suggested this method may be adaptable to measuring 3TC concentrations in plasma and urine. However, it has only been used to monitor 3TC concentrations in serum of HIV-1-infected patients.

Hoetelmans et al. [33] developed a method for determining 3TC concentrations in plasma, saliva, and CSF, and urine. Standards (10–5000 ng/ml 3TC) were made in all three matrices. Sample size was 500 μl for plasma and CSF analysis and 600 μl for saliva analysis. Samples were cleaned via SPE, injected onto an HPLC system (phenyl analytical column, phosphate buffer–methanol, 92:8, v/v, at 1 ml/min), and monitored at 270 nm. Recovery of 3TC by SPE from plasma, CSF, and saliva was 97, 112, and 72%, respectively. Hoetelmans et al. [33] used blank saliva, plasma, and CSF, as well as a variety of nucleoside and non-nucleoside drugs to ensure chromatographic specificity. They also demonstrated the stability of 3TC under various conditions: 1 h at 60°C, 24 h at room temperature, 7 days at 4°C, 30 days at −30°C, and three freeze–thaw cycles. Recovery of 3TC from plasma following each of these conditions was at least 91% of untreated samples. The was no report concerning 3TC stability in saliva or CSF. The only drawbacks to this assay were that no internal standard was used and that the run time was relatively long (50 min).

Error, within-day coefficient of variation, and between-day coefficient of variation were less than 10% throughout the calibration range in plasma. Hoetelmans did not report the accuracy or precision of 3TC analysis in saliva or CSF. The author provided patient data from plasma and CSF samples only.

3.2.3. Zalcitabine

Hawkins et al. [34] published an HPLC–UV procedure for measuring ddC. Samples were passed through C_{18} SPE cartridges, eluted with methanol, dried, resuspended in water, and further cleaned by ultrafiltration. Finally, the samples were introduced into the HPLC system (C_{18} analytical column, heptafluorobutyric acid in acetonitrile at 2.0 ml/min). ddC and the internal standard, 5′-methyldeoxycytidine, were detected by measuring absorbance at 288 and 306 nm, respectively. Standards, which ranged from 0.04 to 0.6 μ*M* ddC, were prepared in plasma. Sample size was large, 1.5 ml plasma. Because multiple wavelengths were monitored, a variable wavelength UV detector was needed. Hawkins et al. [34] also used the less conventional, old-fashioned peak-height ratios for constructing calibration curves. Integrators capable of calculating peak area have been used in most laboratories for several years. They provided no information regarding the accuracy or precision of this method. Plasma ddC concentrations in samples obtained from an adolescent patient were presented.

3.2.4. Stavudine

Kaul et al. [35] published an HPLC–UV method for measuring d4T in rat and monkey plasma. Briefly, 250 μl of sample were combined with internal standard (thymidine oxetane), introduced onto C_{18} SPE cartridges, eluted with methanol, dried, and reconstituted in HPLC mobile phase. The HPLC system consisted of a C_{18} analytical column and phosphate buffer–methanol (80:20, v/v, at 1 ml/min) as mobile phase. d4T and the internal standard were monitored at 254 nm. Controls (0.1–100 μg/ml) were prepared in rat and monkey plasma. Neither rat nor monkey analysis yielded error, intra-assay coefficients of variation, or inter-assay coefficients of variation greater than 10%. The authors used a diode array detector to demonstrate specificity of the assay. Stability of d4T during storage (−20°C for 21 days) and three freeze–thaw cycles was demonstrated. Recovery, as determined by comparing calibration curves constructed with standards extracted from plasma with calibration curves constructed with unextracted standards in HPLC mobile phase, was 86%. The internal standard was provided by Bristol Myers Squibb. Although only used in rat and monkey studies, it was likely that this method could be adapted for human studies.

Wong and Sawchuk [36] wrote a method to measure d4T in human and rabbit plasma and urine. Like Kaul et al. [35], thymidine oxetane was obtained from Bristol Meyers Squibb and used as the internal standard. Either 1 ml plasma or 100 μl urine was mixed with internal standard. Unlike other authors, Wong and Sawchuk incorporated LLE into analysis of plasma and urine samples. Following addition of 5% isopropyl alcohol in methylene chloride, the extracting solvent, the organic layer was kept, dried, and reconstituted in HPLC mobile phase (phosphate buffer–acetonitrile, 93.5:6.5, v/v). The HPLC system consisted of a C_{18} analytical column that was eluted at 0.75 ml/min. Samples were monitored at 264 nm. Standards were prepared in human plasma (0.005–10 μg/l) or human urine (0.05–100 μg/l). Recovery of d4T from extracted plasma was 46% of d4T recovered from an equivalent unextracted aqueous solution. Recovery from urine was not reported. Between- and within-day precision for measuring d4T in plasma and urine were greater than 89%. Accuracy in both matrices was better than 95% over the calibration range. Again, use of more up-to-date equipment would have enabled the author to construct calibration curves with peak area ratios, rather than peak height ratios. Wong and Sawchuk did not assess the specificity or stability of d4T, although this could have been assumed from other publications. The authors used this method to study the pharmacokinetics of d4T in rabbits.

Burger et al. [37] published a method for quantitation of d4T in human plasma using ddI as the internal standard. In short, 500 μl of sample were mixed with internal standard, cleaned up using silica gel SPE (eluted with methanol, dried, reconstituted in HPLC mobile phase), and analyzed by HPLC–UV. Analysis called for a phenyl analytical column with a

phosphate buffer–methanol mobile phase (90:10, v/v, at 1 ml/min) monitored at 265 nm. Standards (10–10 000 ng/ml) were prepared in plasma. Recovery, the ratio of extracted plasma samples with comparable unextracted aqueous samples, was greater than 0.90. Accuracy was also better than 90%. However, Burger et al. [37] made no mention of either intra-assay or inter-assay precision. d4T was reported to be stable during heating (60°C for 30 min) storage at room temperature for 24 h, and storage at −30°C for 21 days. In fact, over 90% of the original d4T was recovered from plasma samples exposed to these conditions. The authors planned to use this method in a clinical setting. However, at the time Burger et al. [37] published this method, it had only been used to study rat plasma. Since ddI was used as internal standard, this method would not be appropriate for analyzing samples from patients receiving ddI therapy.

3.2.5. Didanosine

Carpen et al. [38] suggested an HPLC–UV method for detecting ddI in human plasma, CSF, and urine. The commercially available internal standard, 2′-deoxyguanosine, was added to 1 ml of sample. The plasma and urine samples were then subjected to cleanup via C_{18} SPE cartridges (eluted with methanol). SPE eluent was dried, reconstituted in water, and introduced onto the HPLC system. Recovery of ddI from extracted samples was 85% of non-extracted aqueous samples. CSF samples were injected directly onto the HPLC system (5% acetonitrile in heptafluorobutyric acid (0.1%, v/v) in water at 2 ml/min). As a means of ensuring peak purity, effluent was monitored at 252 and 260 nm. Unfortunately, Carpen's choice of analytical column was unclear. There was no information regarding the error, precision or range or standards. Although urine and plasma samples have been analyzed by this method, ddI was not detected in CSF.

Wientjes and Au [39] used C_{18} SPE (methanol–phosphate buffer, 75:25, v/v, elution) for sample cleanup in his method for determining ddI concentrations in 100 μl of rat plasma. Rat urine was simply diluted and injected directly onto the HPLC system (C_{18} analytical column, phosphate buffer–acetonitrile, 96:4, v/v, at 2 ml/min). 5′-deoxy-5-fluorouridine, a gift from Hoffmann LaRoche, was the internal standard. SPE extraction efficiency was greater than 90%. A diode array detector was used to ensure endogenous peaks would not interfere with ddI analysis. Standards were prepared in both plasma (0.2–200 μg/ml) and urine (0.5–200 μg/ml). However, no other method validation information was presented in the method. Wientjes and Au analyzed rat samples using this procedure.

Ravasco et al. [40] used his method to measure ddI in macaque plasma and urine. Analysis of urine (100 μl) simply required dilution with an aqueous internal standard solution (3′-hydroxyacetamidophenol, Aldrich) before injection onto the HPLC system. Plasma samples (50–400 μl) were mixed with internal standard, eluted from C_{18} SPE cartridges with methanol, dried, reconstituted in HPLC mobile phase, and injected onto the HPLC system. The HPLC system consisted of an acetonitrile–ammonium phosphate (6:94, v/v) mobile phase monitored at 254 nm. Urine standards (0.25–2 μg/ml) were prepared in water and plasma standards (0.025–0.25 μg ddI/sample) in macaque plasma. Plasma standards were not uniform. Instead, they were constructed based upon sample size. Peak height ratios, not peak area ratios were used to construct calibration curves. Again, modern instrumentation would have allowed for peak area analysis. Inter-assay variability, determined by comparing the slope of calibration curves calculated from different analytical runs, was 5.5%. Intra-assay variability, differences in peak height ratio, was less than 10% in both urine and plasma. Accuracy was greater than 90%. Ravasco analyzed plasma and urine samples from macaque using this method. This method may be useful in human studies.

Rosell-Rovira et al. [41] published a method for quantifying ddI in human serum that used ultrafiltration for sample cleanup. The ultrafiltrate was injected directly onto the HPLC system (phenyl analytical column, sodium citrate–isopropanol, 97.5:2.5, v/v, mobile phase, and 250 nm detection). No internal standard was used. Sample size was 250 μl. Recovery of ddI in serum from ultrafiltration was greater than 90%. The authors demonstrated ddI was stable during 8.5 months at room temperature, at 4°C, and at −20°C. Within-day and between-day precision were both greater than 10% throughout the calibration range (25–3000 ng/ml in serum). Drug

interferences were not detected in plasma spiked with NRTIs. Rosell-Rovira et al. analyzed clinical samples from patients receiving ddI therapy.

3.2.6. Abacavir

Veldkamp et al. [42] designed an HPLC–UV method for measuring ABC. Sample cleanup consisted of perchloric acid protein precipitation followed by centrifugation and direct injection of the supernatant onto the HPLC system (C_{18} analytical column at 41°C, phosphate buffer–acetonitrile, 85:15, v/v, at 1 ml/min). Analysis required 300 μl plasma, and absorbance was monitored at 285 nm. Accuracy, within-day precision and between-day precision were greater than 90% throughout the calibration range (20–2000 ng/ml plasma). Recovery of ABC in plasma was 88.1% of unextracted aqueous solutions. Recoveries of ABC from samples subjected to storage for 51 days at −20°C, heating for 4.5 h at 60°C, and four freeze–thaw cycles were within 15% of freshly prepared standards, ensuring ABC stability under these circumstances. Specificity was determined by analyzing aqueous solutions of many common drugs, including NRTIs. Either the drugs had different retention times or they were not detected. No internal standard was used. The author admitted that a small interfering peak was not always separated from ABC. Perhaps better chromatography would prevent co-elution of interfering extraneous peaks with ABC. This method has been used to study ABC in HIV-1-infected patients.

3.2.7. Zidovudine, lamivudine, stavudine, didanosine, and abacavir

Aymard et al. [43] discussed a method for measuring concentrations of amprenavir, efavirenz, indinavir, nelfinavir, ritonavir, saquinavir, nevirapine, ABC, 3TC, ZDV, d4T, and ddI in 1 ml of plasma. The plasma sample was passed through C_{18} SPE cartridges and eluted with methanol. The eluent was divided for use in two separate methods, one for the simultaneous determination of amprenavir, efavirenz, indinavir, nelfinavir, ritonavir, and saquinavir concentrations and the other for the simultaneous determination of nevirapine, ABC, 3TC, ZDV, and d4T concentrations. The aliquot designated for NRTI analysis was injected onto a complicated system that used three different mobile phases to mimic a gradient. Clearly, a binary pump would have simplified the instrumentation. In general, the organic nature of the mobile phase (acetonitrile–phosphate buffer with OSA as an ion pairing agent) was increased during the analysis run and then decreased for re-equilibration before the next analysis. Standards were constructed in plasma and contained all five drugs (20–1000 ng/ml 3TC and ABC and 10–500 ng/ml ddI, d4T, and ZDV). Recovery of 3TC, ddI, d4T, ZDV, and ABC; when extracted plasma sample was compared to direct injection of drug in aqueous solution; was between 70 and 80%. All five drugs were chromatographically separated by more than 1 min (Fig. 2). Specificity was tested by analyzing over 100 compounds in aqueous solution. Only salicylic acid eluted within 0.3 min of an NRTI (ZDV). Absorbance was monitored at 260 nm. Over the range of calibration standards, the within and between coefficients of variation and the error were less than 10% for all NRTIs. There was less than 10% degradation of any of the NRTIs in human plasma stored for 6 months at −20°C. Plasma received from a patient receiving multiple antiretrovirals (including 3TC, d4T, ddI and ABC) was studied using this. Current standard of care involves a therapeutic regimen containing multiple antiretroviral drugs. Thus, methods such as this, capable of measuring a wide spectra of compounds, will help make drug monitoring more efficient.

3.3. Mass spectrometry as the detection step

The selectivity of tandem mass spectrometry results [6,7] from monitoring fragmentation of a parent ion with a specific m/z to a product ion of a specific m/z. Interference can only occur if two or more molecules are introduced into the source simultaneously and fragment to form precursor and product ions of identical m/z. Tandem quadrupole mass spectrometers operated in the multiple reaction monitoring acquisition mode (MRM) are capable of monitoring multiple precursor→product transitions by cycling rapidly through individual precursor→product transitions. MRM is useful for detecting multiple molecules, including analyte and stable isotopically labeled internal standard.

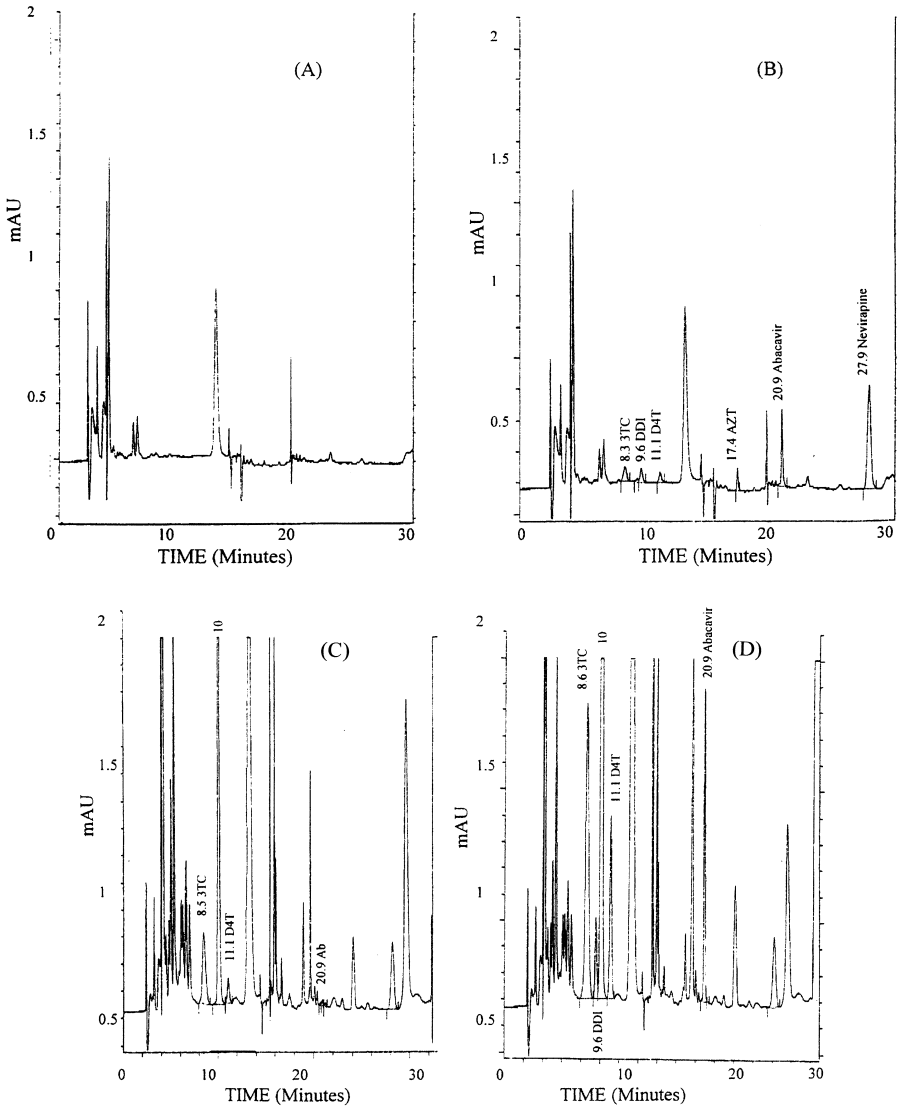

Fig. 2. Separation of zalcitabine, stavudine, zidovudine, lamivudine, and abacavir in human plasma by HPLC–UV. Following sample clean up with SPE, separation of DDI, D4T, ZDV, 3TC, and ABC was achieved by reversed-phase liquid chromatography with UV absorbance detection. Chromatograms represent analysis of drug-free plasma sample (A), spiked plasma sample containing 25 ng/ml DDI, D4T, and ZDV, 50 ng/ml 3TC, and 100 ng/ml ABC (B), clinical sample containing 73 ng/ml D4T, 345 ng/ml 3TC and concentrations below the limits of quantitation of DDI and ABC (C), and clinical sample containing 176 ng/ml DDI, 511 ng/ml D4T, 1430 ng/ml 3TC, and 556 ng/ml ABC (D). This method was capable of separating and quantifying five NRTIs in a single assay with relatively common instrumentation. However, a low signal-to-noise ratio, large sample size, long runtime, and lack of an internal standard may preclude its use at times when small concentrations of drug are to be measured quickly and accurately. Adapted from Ref. [43] with permission.

3.3.1. Zidovudine

Font et al. [44] published a method for the quantitation of intracellular ZDV-TP using HPLC–MS–MS. Calibration standards, consisting of known amounts of ZDV-TP in cell extract, ranged from 4 to 10 000 fmol/10^6 cells. The chosen internal standard, AzdU was commercially available. However, it was not the ideal, stable isotopically labeled ZDV-TP.

Because stable isotopically labeled ZDV-TP has the same chromatographic properties as ZDV-TP, its use would have shortened chromatographic run time and would have corrected for any ionization fluctuations in the ion source. As with other methods used to quantitate intracellular concentrations of NRTI-TPs, 16 ml of whole blood were required. Cell extracts were passed through SAX SPE cartridges, which were then washed with 74.5 m*M* KCl. ZDV-TP was eluted from the column with 1 *M* KCl. AzdU was added to the eluent, and the mixture was incubated with 2 U acid phosphatase for 30 min. Before injection into the mass spectrometer, the authors used an XAD column to desalt the mixture. The eluent was dried and reconstituted in HPLC mobile phase (acetonitrile–methanol, 10:30, with 0.25% acetic acid) and injected onto the HPLC (C_{18} analytical column at a flow of 0.2 ml/min). Finally, the HPLC eluent entered the electrospray source. ZDV-TP and the internal standard were monitored by MRM. Because cells without ZDV incubation have no signal on chromatogram, the author concluded endogenous cell components did not interfere with ZDV-TP analysis. Recovery of [^{3}H]ZDV-TP was greater than 95%. Inter-assay variability and error were both less than 10% throughout the calibration range. Stability of ZDV-TP was not addressed. Data from patient samples and cells incubated with ZDV were presented. This technique is superior to earlier published methods for the quantitation of intracellular ZDV-TP. It required less time, had a lower limit of quantitation, and used ZDV-TP not ZDV to create calibration curves.

3.3.2. Zalcitabine

Jajoo et al. [45] published one of the earliest methods to quantify an NRTI using mass spectrometry. Hoffmann LaRoche provided the internal standard, [$^{15}N_2^2H_2$]ddC. A mixture of internal standard and 1 ml plasma sample was added to C_{18} SPE cartridges and eluted with a methanol–water mixture (20:80, v/v). The eluent was dried under nitrogen, reconstituted in HPLC mobile phase (methanol–0.05 *M* ammonium acetate, 10:90, at 1 ml/min), and injected onto the HPLC. Recovery of ddC from SPE extraction was greater than 90%, as determined by total reactivity. Standards used to generate the calibration curve (0.25–20 ng/ml) were created in water. Perhaps using drug-free plasma, which was readily available, for creating calibration standards would have better mimicked patient samples. The authors gave no report of the method's accuracy or precision. Finally, because only a single quadrupole mass spectrometer was used, the power of tandem triple quadrupole technology was realized. In other words, the method was designed to monitor parent ions [M+H] of ddC and internal standards, rather than monitoring the transitions of ddC and internal standard from parent to product ions. A concentration-time profile of ddC in human plasma was shown.

3.3.3. Zidovudine and lamivudine

More recently, Kenney et al. [46] published a method to quantitate ZDV and 3TC in 25 μl human serum by a single automated procedure using HPLC–MS–MS. Sample preparation was simple, requiring addition of stable isotopically labeled ZDV and 3TC ([$^{13}C^{2}H_3^{15}N$]ZDV and [$^{13}C_2^{15}N_3$]3TC, respectively) and ultrafiltration through a 30 000 MW cut-off membrane. Neither internal standard, however, is commercially available. The ultrafiltrate was injected onto a C_{18} analytical column and eluted with acetonitrile–water (15:85, v/v) at a flow of 0.3 ml/min. The HPLC eluent was directed into a turbo-ion spray source for electrospray ionization. Parent→product transitions of ZDV, 3TC, and their internal standards were monitored using MRM (Fig. 3). Extraction efficiencies were 104% for ZDV and 99.7% for 3TC. Calibration curves (2.5–2500 ng/ml ZDV and 2.5–5000 ng/ml 3TC) were constructed from standards prepared in human serum. Between-day precision, within-day precision, and accuracy were greater than 85% for both drugs over the range of calibration standards. Specificity was determined by analyzing serum from drug-free volunteers, and the authors noted no interferences. Stability studies of ZDV and 3TC (in aqueous stock solution stored at 10°C for 7 months, in ultrafiltrate stored at room temperature for 72 h, in plasma during heat inactivation of HIV-1 for 5 h at 58°C, and in plasma for 6 months at −40°C with three freeze–thaw cycles) revealed no loss of either drug. This method was cross-validated with the commercially available ZDV RIA kit and the HPLC–UV method published by

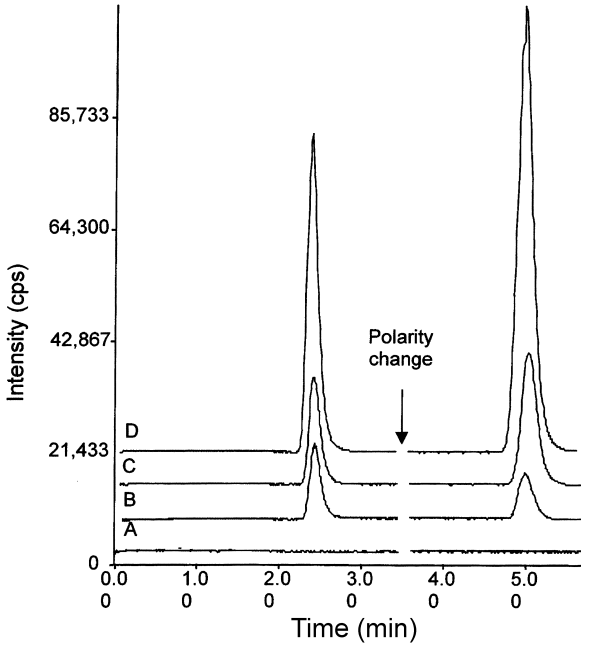

Fig. 3. Separation of zidovadine and lamivudine in human serum by HPLC–MS–MS. After passage through a molecular mass filter, separation of ZDV and 3TC was achieved with the use of reversed-phase liquid chromatography with tandem mass spectrometry. Chromatograms represent parent–product transitions of ZDV (5 min) and 3TC (2.5 min) obtained during analysis of drug-free serum sample (A) and clinical samples containing 20 and 120 (B), 425 and 322 (C), and 1903 and 1308 ng/ml ZDV and 3TC, respectively (D). This method was capable of quantifying ZDV and 3TC in a single assay using stable-labeled internal standards with a relatively short runtime and a small sample volume. Analysis produced chromatograms with good peak separation and a large signal-to-noise ratio. However, until mass spectrometers become more common, this method may be limited to the large research universities and pharmaceutical companies that can afford such expensive instrumentation. First reported in Ref. [46] (with permission).

Harker et al. [30]. It was more sensitive, faster, and required less sample than the RIA or HPLC–UV methods. This method has been used to support clinical studies of Combivir, a single tablet of ZDV and 3TC.

Pereira et al. [47] developed and validated an HPLC–MS–MS method to quantify ZDV and 3TC in human seminal plasma. Sample preparation, choice of internal standards and HPLC system were all based on the method developed by Kenney et al. [46]. Like Kenney et al., sample size was 25 μl. Calibration standards were constructed bull seminal plasma and ranged from 5 to 10 000 ng/ml of both ZDV and 3TC. Bull semen, rather than human semen, was chosen because it could be obtained at a lower cost. Controls were created in human seminal plasma to ensure bull semen was an appropriate substitution. Between-day precision, within-day precision, and accuracy were greater than 85% throughout the range of calibration standards. The authors noted no interference from seminal samples collected from drug-free healthy humans. Stability testing revealed no difference between the concentration of ZDV and 3TC in seminal standards stored for 9 months at −5°C and freshly prepared seminal standards. This method has been used in clinical setting to measure ZDV and 3TC in the seminal plasma of men receiving these drugs as part of their antiretroviral therapy.

Rodriguez et al. [48] designed a method for determining intracellular concentrations of multiple NRTI-TP's. The required sample size to measure intracellular concentrations of both ZDV-TP and 3TC-TP was 16 ml whole blood. This method was very similar to one published by Font et al. [44] for ZDV-TP quantitation. Cell extracts were passed through ZAX-QMA cartridges and parent drugs were eluted with water. ZDV-MP and 3TC-MP were eluted with 100 m*M* KCl, ZDV-DP and 3TC-DP with 120 m*M* KCl, and ZDV-TP and 3TC-TP with 400 m*M* KCl. Each fraction (-MP, -DP, -TP) was incubated with 2 U acid phosphatase for 30 min. The internal standard, AzdU, was then added to each fraction. The authors used an XAD column to desalt the fractions before MS–MS analysis. The desalted fractions were injected onto a C_{18} analytical column and eluted with a methanol–acetonitrile mobile phase (30:10, v/v, with 0.25 acetic acid) at a flow of 0.2 ml/min. MRM was used to monitor ZDV-TP, 3TC-TP, and the internal standard. Standards were constructed by adding 3TC-TP and ZDV-TP to PBMC (peripheral blood mononuclear cell) extract. Recoveries of both ZDV-TP and 3TC-TP, as compared to [^{3}H]ZDV-TP and [^{3}H]3TC-TP, were both greater than 95%. Analysis of ZDV-TP and 3TC-TP had an error less than 10% and the coefficent of variation of within-day analysis was also less than 10%. Between-day precision was not reported. Data

from patient samples collected throughout the dosing interval was presented as a means to understand the intracellular pharmacokinetic profiles of ZDV-TP and 3TC-TP.

3.3.4. Stavudine, didanosine, or abacavir

No published method for quantifying d4T, ddI, or ABC by mass spectrometry have been published. Because popularity of this analytical tool is growing, it is likely that more mass spectrometry methods will be published.

4. Conclusions

Before choosing a method from those reviewed here (Table 1), one should consider the equipment available, analyte to be measured, and sample matrix. Once chosen, the method should be re-validated by the end user. Often minor reagent substitutions, changes in laboratory temperature, or lot-to-lot variations in extraction cartridges and analytical columns affect analytical results. Sometimes these changes can be detrimental, other times beneficial. We, as researchers, must continue to share and publish our analytical improvements so that eventually patient care might also be improved.

Currently, researchers use immunoassays, HPLC–UV methods, and HPLC–MS–MS methods to study NRTIs and their metabolites. In this author's opinion, HPLC–MS–MS will become the method of choice for most analysts. Currently, both the instrument cost and the required level of technical expertise prevent routine use of HPLC–MS–MS analyses. However, we expect as more researchers purchase mass spectrometers and develop HPLC–MS–MS methods, instrumentation costs will lower and the technology will become more user-friendly. In general, HPLC–MS–MS methods are fast, allow the use of isotopically internal standards, and are capable of monitoring multiple compounds in a single assay without laborious sample preparation. All will be important in the future, as more clinicians therapeutically monitor their HIV-1-positive patients who are receiving multiple NRTIs.

5. Nomenclature

3TC	Lamivudine
ABC	Abacavir
AIDS	Acquired immunodeficiency syndrome
AMT	3′-Amino-3′-deoxythymidine
CSF	Cerebral spinal fluid
d4T	Stavudine
ddI	Zalcitabine
-DP	Diphosphate
ELISA	Enzyme-linked immunosorbent assay
FPIA	Fluorescence polarization immunoassay
HIV-1	Human immunodeficiency virus type-1
HPLC	High-performance liquid chromatography
HPLC–MS	High-performance liquid chromatography coupled with mass spectrometric detection
HPLC–MS–MS	High-performance liquid chromatography coupled with tandem mass spectrometric detectors
HPLC–UV	High-performance liquid chromatography coupled with ultraviolet absorbance detection
IgG	Immunoglobulin G
LLE	Liquid– liquid extraction
MP	Monophosphate
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS–MS	Tandem mass spectrometry (tandem quadropole mass spectrometry)
NRTI	Nucleoside reverse transcriptase inhibitor
NRTI-TP	Triphosphorylated nucleoside reverse transcriptase inhibitors
OPD	*o*-Phenylene dihydrochloride
PBMC	Peripheral blood mononuclear cell
PPL	Plane polarized light
RIA	Radioimmunoassay
SRM	Single (selected) reaction monitoring
SPE	Solid-phase extraction

Table 1
Summary of reviewed analytical methods

Author	Analyte	Matrix	Method
Quinn et al. [9]	ZDV	H-Plasma	RIA
Tadepalli et al. [10]	ZDV ZDV-G	H-Urine H-Serum	RIA
Stretcher et al. [11]	ZDV ZDV-TotP	H-Whole blood	RIA
Kuster et al. [12]	ZDV-MP ZDV-DP ZDV-TP	H-Whole blood	HPLC–SPE–RIA
Slusher et al. [13]	ZDV-MP ZDV-DP ZDV-TP	H-Whole blood	HPLC–SPE–HPLC–RIA
Peter et al. [14]	ZDV-MP ZDV-DP ZDV-TP	H-Whole blood	HPLC–SPE–RIA
Robbins et al. [15]	ZDV-MP ZDV-DP ZDV-TP	H-Whole blood	SAX SPE–SPE–RIA
Robbins et al. [16]	3TC-TP ZDV-TP	H-Whole blood	SPE–RIA
Roberts et al. [17]	ddC	H-Plasma	SCX SPE–RIA
Kastrissios et al. [18]	ddC	H-Plasma	SPE–RIA
Zhou et al. [19]	d4T	H-Plasma	RIA
DeRemer et al. [20]	ddI	H-Plasma	RIA
Tadepalli and Quinn [21]	ZDV	H-Serum	LLE–ELISA
Ferrua et al. [22]	d4T	H-Serum	ELISA
Granich et al. [23]	ZDV	H-Serum	PP–FIPA
Unadkat et al. [24]	ZDV	H-Plasma H-Urine	LLE–HPLC–UV
Hedaya and Sawchuk [25]	ZDV	H-Plasma H-Urine	LLE–HPLC–UV
Good et al. [26]	ZDV ZDV-G	H-Serum	SPE–HPLC–UV
Nadal et al. [27]	ZDV ZDV-G	H-Plasma	SPE–HPLC–UV
Ruprecht et al. [28]	ZDV	Mou-Brain Mou-Embryonic tissue Mou-Milk Mou-Serum	PP–HPLC–UV
Burger et al. [29]	AMT	H-Plasma	SCX SPE–HPLC–UV
Harker et al. [30]	3TC	H-Serum	SPE–HPLC–UV

Table 1. Continued

Author	Analyte	Matrix	Method
Morris and Selinger [31]	3TC	H-Urine	HPLC–Column Switching-UV
Zhou and Sommadossi [32]	3TC	H-Serum	PP–HPLC–UV
Hoetelmans et al. [33]	3TC	H-Plasma H-Saliva H-CSF H-Urine	SPE–HPLC–UV
Hawkins et al. [34]	ddC	H-Plasma	SPE–UF–HPLC–UV
Kaul et al. [35]	d4T	Rat-Plasma Mon-Plasma	SPE–HPLC–UV
Wong and Sawchuk [36]	d4T	H-Plasma H-Urine Rab-Plasma Rab-Urine	LLE–HPLC–UV
Burger et al. [37]	d4T	H-Plasma	Gel SPE–HPLC–UV
Carpen et al. [38]	ddI	H-Plasma	SPE–HPLC–UV
Wientjes and Au [39]	ddI	Rat-Plasma Rat-Urine	SPE–HPLC–UV
Ravasco et al. [40]	ddI	Mon-Plasma Mon-Urine	SPE–HPLC–UV
Rossell-Rovira et al. [41]	ddI	H-Serum	UF–HPLC–UV
Veldkamp et al. [42]	ABC	H-Plasma	PP–HPLC–UV
Aymard et al. [43]	ZDV 3TC d4T ddI ABC	H-Plasma	SPE–HPLC–UV
Font et al. [44]	ZDV-TP	H-Whole blood	SAX SPE–XAD–HPLC–MS–MS
Jajoo et al. [45]	ddC	H-Plasma	SPE–HPLC–MS
Kenney et al. [46]	ZDV 3TC	H-Serum	UF–HPLC–MS–MS
Pereira et al. [47]	ZDV 3TC	H-Semen	UF–HPLC–MS–MS
Rodriguez et al. [48]	ZDV-TP 3TC-TP	H-Whole blood	SAX SPE–XAD–HPLC–MS–MS

ZDV-TotP, total ZDV-phosphates; H, Mou, Rat, Mon, and Rab, human, mouse, rat, monkey, and rabbit; SPE, LLE, PP, UF designate methods requiring solid-phase extraction, liquid–liquid extraction, protein precipitation, or ultrafiltration for sample cleanup.

TP	Triphosphate
UV	Ultraviolet (also ultraviolet absorption)
ZDV	Zidovudine
ZDV-G	Glucuronidated ZDV

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