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# Sensitive and rapid liquid chromatography–tandem mass spectrometry method for the determination of stavudine in human plasma

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**Abstract**

A sensitive method for the determination of stavudine in plasma was developed, using high-performance liquid chromatographic separation with tandem mass spectrometric detection. The samples were extracted from plasma with Waters, Sep-Pak®Vac, 100 mg, $tC_{18}^{®}$ solid-phase extraction (SPE) columns. Chromatography was performed on a Supelco Discovery® $C_{18}$, 5 μm, 150×2 mm column with a mobile phase consisting of ammonium acetate (0.01 *M*)–acetonitrile–methanol (800:100:100, v/v/v) at a flow-rate of 0.3 ml/min. Detection was achieved by an Applied Biosystems API 2000 mass spectrometer (LC–MS–MS) set at unit resolution in the multiple reaction monitoring mode (MRM). Atmospheric pressure chemical ionization (APCI) was used for ion production. The mean recovery for stavudine was 94% with a lower limit of quantification set at 4 ng/ml. This assay method makes use of the increased sensitivity and selectivity of mass spectrometric (MS–MS) detection to allow for a more rapid (extraction and chromatography) and selective method for the determination of stavudine in human plasma than has previously been described. 

*Keywords:* Stavudine

## 1. Introduction

Stavudine (2′,3′-didehydro-3′ deoxythymidine, d4T, Zerit®) is a thymidine analogue with in vitro and in vivo activity against the human immunodeficiency virus (HIV). It is a reverse transcriptase inhibitor whose mode of action is similar to that of other nucleoside analogues and is active at concentrations that are generally 100-fold below the levels which are cytotoxic. Following phosphorylation by cellular kinases, d4t-triphosphate is produced, which preferentially inhibits HIV-1 reverse transcriptase activity [1–4]. Moore et al. [8] describe a sensitive LC–MS–MS method for the simultaneous measurement of the intracellular nucleoside 5′-triphosphate anabolites of zidovudine, lamivudine and stavudine in peripheral blood mononuclear cells which are the sites of HIV replication and drug action. An oral dose of stavudine (40 mg) leads to a maximum plasma stavudine concentration of around

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876 ng/ml after 90 min [6]. In this study we determined the pharmacokinetics of stavudine up to 24 h after a single oral dose of 40 mg stavudine.

Several methods have been described for the determination of stavudine in plasma. The most widely used methods involve high-performance liquid chromatography (HPLC) with ultraviolet detection achieving lower limits of quantification (LLOQ) of around 10–25 ng/ml [6,7]. Kaul et al. [5] achieved the best sensitivity with an LLOQ of 2.5 ng/ml for stavudine using a radioimmunoassay (RIA) method. We initially developed an assay method using HPLC with UV detection but due to a very aqueous mobile phase and a common UV detection wavelength, too much interference from endogenous components was observed. To overcome this problem, very long chromatography times were required to prevent late eluting peaks from interfering with the analyte of interest. This made the method impractical for the determination of large numbers of samples. It was therefore decided to develop a new method involving the use of a mass-selective detector with mass spectrum–mass spectrum (MS–MS) capabilities in tandem with liquid chromatography (LC) to increase the selectivity which would allow for more rapid chromatography and sample clean-up. This report describes an LC–MS–MS method for the determination of stavudine in plasma using a simple solid-phase extraction procedure. With a total turnaround of 4 min between sample injections, the analyst is able to assay a large number of samples per day. The LLOQ of 4 ng/ml is also sensitive enough to do pharmacokinetic studies after a 40-mg oral dose of stavudine.

## 2. Experimental

### 2.1. Materials and chemicals

A Supelco Discovery® $C_{18}$ 5 μ, 2.1×150 mm column (Supelco, Bellefonte, PA, USA) was used for separation at a flow-rate of 0.3 ml/min and injecting 20 μl onto the column. The mobile phase was delivered by an Agilent Series 1100 pump (Agilent, Palo Alto, CA, USA) and the samples injected by an Agilent Series 1100 autosampler. Detection was performed by an Applied Biosystems API-2000 detector (Applied Biosystems, Ontario, Canada) using atmospheric pressure chemical ionisation (APCI) for ion production.

Methanol and acetonitrile (Burdick and Jackson, High Purity) were obtained from Baxter chemicals, USA; sodium hydroxide and ammonium acetate were obtained from Fluka chemicals (Buchs, Switzerland). Orthophosphoric acid (85%) was obtained from Merck (Darmstadt, Germany). All chemicals were used as received. Water was purified by Millipore Elix 5 reverse osmosis and Milli-Q® (Millipore) Gradient A10 polishing system (Millipore, Bedford, MA, USA). Phosphate buffer (0.05 *M*) was prepared from 0.05 *M* phosphoric acid and adjusted to pH 7 with sodium hydroxide (5 *M*).

Stavudine ($C_{10}H_{12}N_2O_4$) was supplied by Cipla Ltd., Mumbai Central, India.

### 2.2. Extraction procedure

A stavudine standard solution was made up in water and used immediately to spike plasma and discarded thereafter. Calibration standards and quality control standards were prepared in normal human plasma by spiking a pool of normal human plasma which was then serially diluted with normal blank plasma to attain the desired concentrations (4–2029 ng/ml for the calibration standards and 5–1625 ng/ml for the quality control standards). The calibration standards and quality control standards were aliquoted into Cellstar® Cryo.s tubes and stored under the same conditions as the trial samples; at −20 °C. Stavudine has been shown to be stable at −20 °C for at least 1 year [5].

The plasma samples were thawed in a waterbath at ~37 °C, mixed for 5 s on a vortex mixer and centrifuged for 5 min at 3000 *g*. A solid-phase extraction (SPE) procedure was then performed manually, using Waters, Sep-Pak®Vac, 100 mg, t$C_{18}$® SPE columns and a SPEEDISK® 48 plate positive pressure system. The SPE columns were conditioned with methanol (1 ml), water (1 ml) and a phosphate buffer (1 ml, 0.05 *M*, pH 7). The samples were then loaded (0.5 ml plasma diluted with 0.5 ml phosphate buffer) onto the SPE columns. The columns were rinsed with phosphate buffer (1 ml, 0.05 *M*, pH 7) and water (1 ml). The analyte was eluted with methanol (0.5 ml) and the eluate evaporated to dryness using a Savant SpeedVac® rotary concentrator (~60 °C) for ~90 min. The residual extracts

were redissolved in water (0.25 ml) by mixing on a vortex mixer for 30 s. The samples were transferred into autosampler vials and 20 μl were injected onto the HPLC column.

### 2.3. Liquid chromatography

All chromatographic solvents were degassed with helium before use. Chromatography was carried out at ambient temperature with a mobile phase consisting of ammonium acetate (0.01 *M*)–acetonitrile–methanol (800:100:100, v/v/v) at a flow-rate of 0.3 ml/min.

### 2.4. Mass spectrometry

Atmospheric pressure chemical ionization was performed in the negative ion mode with nitrogen as the nebulizing, turbo spray and curtain gas with the optimum values set at 70, 20 and 40 (respective arbitrary values). The heated nebulizer temperature was set at 450 °C and the nebulizer current on −3.0 μA. The instrument response was optimised for stavudine by infusing a constant flow of a solution of the drug dissolved in mobile phase via a T-piece into the stream of mobile phase eluting from the column. The pause time was set at 5 ms and the dwell time at 150 ms.

Atmospheric pressure chemical ionization (APCI) was used for ion production and the collision gas ($N_2$) set at 3 (arbitrary value).

The Applied Biosystems API 2000 LC–MS–MS detector was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the deprotonated molecular ion *m*/*z* 223.1 to the product ion *m*/*z* 42.01. Fig. 1 shows the single parent (*m*/*z* 223.1) to product ion MS–MS of stavudine. The molecular structure for the analyte is also indicated in this figure.

The instrument was interfaced with a computer running Applied Biosystems Analyst version 1.0 software.

### 2.5. Validation

The method was validated by analysing plasma quality control samples six times at nine different

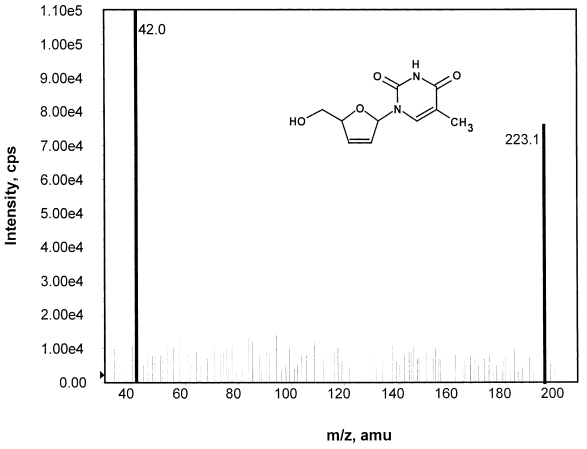


Fig. 1. Full mass spectrum of the deprotonated stavudine molecular ion (*m*/*z* 223.1, molecular structure given) and the principal product ion formed at *m*/*z* 42.01 after collision (MS–MS).

stavudine concentrations, i.e. 1625, 813, 406, 203, 102, 50.8, 19.3, 9.65 and 4.83 ng/ml to determine the accuracy and precision of the method. The quality control values were calculated from a standard regression curve containing 10 different concentrations spanning the concentration range (2029–3.97 ng/ml). Calibration graphs were constructed using a weighted linear regression (1/concentration$^2$) of the drug peak-area of the product ions versus nominal drug concentrations. Several regression types were tested and the weighted linear regression (1/concentration$^2$) was found to be the simplest regression, giving the best results.

The matrix effect (co-eluting, undetected endogenous matrix compounds that may influence the analyte ionisation) was investigated by extracting ''blank'' normal human plasma from 10 different sources, reconstituting the final extract in injecting solvent containing a known amount of the analyte, analysing the reconstituted extracts and then comparing the peak areas of the analytes.

Absolute recoveries of the analyte were determined in triplicate in normal plasma by extracting drug-free plasma samples spiked with stavudine. Recoveries were calculated by comparison of the analyte peak-areas of the extracted samples with those of the unextracted system performance verification standard mixtures (prepared in the injection vehicle) representing 100% recovery.

Table 1
Intra-day quality control results of stavudine

| Nominal concentration (ng/ml) | Stavudine ($n=9$) Mean concentration found (ng/ml) | RSD (%) | % Nominal |
|---|---|---|---|
| 1625 | 1701 | 4.8 | 104.6 |
| 813 | 827 | 2.8 | 101.7 |
| 406 | 410 | 2.0 | 101.1 |
| 203 | 199 | 6.3 | 97.9 |
| 102 | 105 | 8.2 | 102.5 |
| 50.8 | 51.9 | 5.7 | 102.2 |
| 19.3 | 19.6 | 4.0 | 101.7 |
| 9.65 | 10.0 | 3.9 | 103.6 |
| 4.83 | 4.92 | 2.5 | 101.8 |

## 3. Results and discussion

The mean absolute recoveries of stavudine determined in triplicate at 1625, 406 and 102 ng/ml were 96% (RSD 5.1%), 93% (RSD 2.2%) and 94% (RSD 9.0%), respectively. No matrix effect for stavudine was observed for 10 different plasma pools tested. The peak areas of the 10 reconstituted samples had a coefficient of variation of 3.5% indicating that the extracts were ''clean'' with no co-eluting compounds influencing the ionisation of the analyte.

Initially a HPLC method using UV detection was developed. Due to the highly polar nature of stavudine a very aqueous mobile phase had to be used leading to many late eluting peaks. Different columns were tested with a phenyl-hexyl column giving the best results (retention, peak shape). Under these conditions the chromatography time had to be in excess of 25 min which made the method very time-consuming and not very productive. The much higher selectivity of MS–MS detection allowed the development of a very specific and rapid method for the determination of stavudine in plasma.

The LLOQ, defined as that concentration of stavudine which can still be determined with acceptable precision (C.V.%$<$20) and accuracy (bias $<$20%) was found to be 4 ng/ml. Results of the intra-day and inter-day validation assays presented in Tables 1–3 indicate a valid calibration range of 2029–4 ng/ml for stavudine.

On-instrument stability was inferred from stability samples which were prepared and included in the validation batch. No significant degradation could be detected in the samples (ambient temperature) left on the autosampler for at least 49 h.

Due to the high specificity of MS–MS detection, no interfering or late eluting peaks were found when chromatographing blank plasma extracts from six different sources.

Several extraction procedures were tested which included protein precipitation, solid-phase and liquid–liquid extraction methods. A solid-phase ex-

Table 2
Inter-day 1 quality control results of stavudine

| Nominal (ng/ml) | 4.83 | 9.65 | 19.3 | 406 | 813 | 1625 |
|---|---|---|---|---|---|---|
| Mean | 4.43 | 9.07 | 19.2 | 402 | 822 | 1683 |
| RSD | 3.6 | 5.2 | 6.3 | 5.7 | 4.1 | 6.0 |
| % Nominal | 91.7 | 94.0 | 99.2 | 99.1 | 101.1 | 103.5 |
| *n* | 6 | 6 | 6 | 6 | 6 | 6 |

Table 3
Inter-day 2 quality control results of stavudine

| Nominal (ng/ml) | 4.83 | 9.65 | 19.3 | 406 | 813 | 1625 |
|---|---|---|---|---|---|---|
| Mean | 4.96 | 9.35 | 18.5 | 390 | 764 | 1646 |
| RSD | 4.9 | 7.7 | 6.5 | 3.8 | 7.0 | 5.0 |
| % Nominal | 102.8 | 96.8 | 95.8 | 96.1 | 94.0 | 101.3 |
| *n* | 6 | 6 | 6 | 6 | 6 | 6 |

traction procedure proved to be the most successful. The extracts were clean with high recovery rates.

Different concentrations of ammonium acetate were tested for optimum ionisation of the analyte and it was found that 10 m*M* ammonium acetate gave the best result. Stavudine also gave a much higher (10-fold) response with APCI than with electrospray ionisation (ESI).

A plausible fragmentation mechanism resulting in the strong low mass negative product ion of $m/z$ 42 monitored in this assay method is presently under investigation.

Due to the unavailability of a deuterated analogue of stavudine or other suitable internal standards, it was decided to work without an internal standard. Two sets of calibration and quality control standards were used (one set in the first half and one set in the second half) in each batch of 215 samples arranged to compensate for any temporal change in the ionisation response. The ionisation response, monitored by injecting a system performance verification standard at the beginning and at the end of each batch indicated that the system response remained stable with the response not varying more than 5% within each batch.

The retention time for stavudine was 1.97 min. A total chromatography run time of 4 min made it possible to analyse large batches of samples (215 samples) per day. Fig. 2 shows a representative chromatogram obtained of stavudine at a concentration of 4 ng/ml (the LLOQ) and of a study sample close to the limit of quantification at the late elimination phase of the pharmacokinetic profile for the analyte.

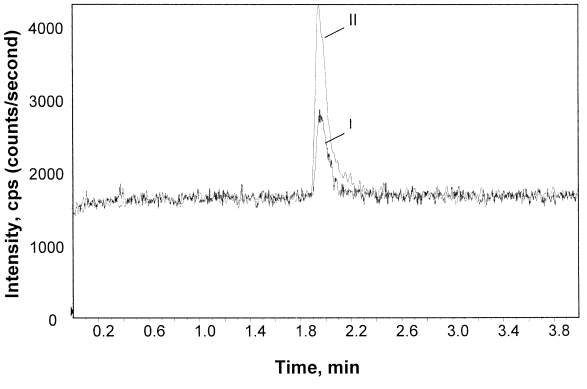


Fig. 2. High performance liquid chromatograms of the calibration standard at the limit of quantification (I) containing 4 ng/ml stavudine and of a study sample (II) close to the limit of quantification at the late elimination phase of the pharmacokinetic profile of the analyte.

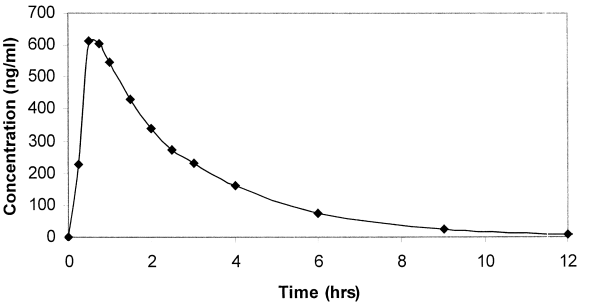


Fig. 3. Representative stavudine plasma concentrations vs. time profiles as obtained after a single 40 mg oral dose of stavudine (24 subjects).

The method was employed to analyse plasma samples containing stavudine obtained after a single oral dose of 40 mg stavudine per treatment phase in 24 healthy volunteers. Concentration vs. time profiles were constructed for up to 24 h for the analyte (Fig. 3).

The maximum stavudine plasma concentrations obtained varied between 399 and 1435 ng/ml. Stavudine was very rapidly absorbed leading to maximum plasma concentrations being reached within 1 h. The elimination half-life of stavudine was 2.25 h.

## 4. Conclusion

A rapid, sensitive and highly selective method for the determination of stavudine in plasma was developed, using high-performance liquid chromatographic separation with tandem mass spectrometric detection. With an LLOQ of 4 ng/ml, pharmacokinetic profiles of the drug could be constructed for up to 24 h after a single oral dose of 40 mg stavudine. The method is more selective than previously described methods and allows for a much higher sample throughput due to the short chromatography time (4 min) and relatively simple sample preparation.

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