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Determination of serum levels of thirteen human immunodeficiency virus-suppressing drugs by high-performance liquid chromatography

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Abstract

Two high-performance liquid chromatographic methods using UV detection are presented for the determination of two different groups (A and B) of drugs used in the suppression of human immunodeficiency virus (HIV). Group A is comprised of six nucleosidic reverse transcriptase inhibitors, viz. zalcitabine, lamivudine, stavudine, didanosine, zidovudine and ziagen. Group B consists of seven drugs four of which are protease inhibitors (PIs) and three of which are non-nucleosidic reverse transcriptase inhibitors (NNRTIs). The PIs are: indinavir, nelfinavir, saquinavir and ritonavir. The NNRTIs are: nevirapine, delavirdine and efavirenz. Groups A and B require separate aliquots of serum for extraction and must be chromatographed separately.

Keywords: Human immunodeficiency virus; Enzyme inhibitors

1. Introduction

It is common practice to initiate treatment of acquired immune deficiency syndrome (AIDS) patients with one protease inhibitor (PI) and two or more reverse transcriptase inhibitors simultaneously [1]. The reverse transcriptase inhibitors may be nucleosidic (NRTIs) or non-nucleosidic reverse transcriptase inhibitors (NNRTIs). These three drug classes have different mechanisms of action and the use of drugs from different classes hinders the development of cross drug resistance. Though there are many different drug strategies used, or in development, multiple-drug therapy is the rule.

Because of the use of multiple drugs in the same patient analytical methods are needed for simultaneously determining blood levels for as many anti-human immunodeficiency virus (anti-HIV) drugs as possible. A clinician may use such methods to provide valuable information about patient treatment in several regards: malabsorption, drug interactions, compliance, individual drug kinetics, and therapeutic drug level monitoring. Though medical evidence suggests that only PIs may show good correlation between drug levels and drug efficacy [2], blood levels may still be of interest to determine factors such as compliance and drug exposure. Drugs present at levels below the therapeutic range may encourage the development of drug resistant strains of HIV by natural selection.

Most analytical methods described in the literature to date involve single drugs [3] or, for studies of drug interactions, pairs of drugs [4]. An important exception is the work of Remmel et al. [5] in which the simultaneous analysis of four of the currently marketed PIs, indinavir, nelfinavir, ritonavir and saquinavir, is reported. The method described by Remmel et al. involves liquid–liquid extraction,

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whereas the present method includes more drugs and uses solid-phase extraction (SPE).

2. Experimental

2.1. Chemicals and supplies

Solvents, acetonitrile, high-performance liquid chromatography (HPLC)-grade water, and methanol, were purchased from Fisher Scientific (Norcross, GA, USA). 5 *M* Sulfuric acid was reagent grade and was also from Fisher Scientific. Zidovudine (AZT) was purchased from Sigma (St. Louis, MO, USA). Nevirapine was a gift from Boehringer Ingelheim (Ridgefield, CT, USA). Indinavir sulfate was a gift from Merck (Rahway, NJ, USA). Nelfinavir mesylate was a gift from Agouron (La Jolla, CA, USA). Saquinavir mesylate was a gift from Hoffmann-La Roche (Nutley, NJ, USA). Ritonavir was a gift from Abbott Labs. (Abbott, IL, USA). All of these companies are sincerely thanked for their generous contributions. Delavirdine made by Upjohn (Kalamazoo, MI, USA) and efavirenz made by Merck were supplied in the form of pills and capsules, respectively, from the pharmacy of the A.G. Holley Hospital (Lantana, FL, USA).

Nylon filters were from Gelman (Ann Arbor, MI, USA). Extrasep C_{18} cartridges were from Lida (Kenosha, WI, USA). Dual-zone C_{18} SPE cartridges were supplied by Diazem (Midland, MI, USA). SPE cartridges were connected to syringes by use of a sample reservoir adapter (Supelco, Bellefonte, PA, USA). Chromatography was carried out using a Luna column supplied by Phenomenex (Torrance, CA, USA).

2.2. Instrumentation

Samples were injected using a Perkin-Elmer ISS-200 autosampler (Perkin-Elmer, Norwalk, CT, USA). The analytical pump was a Perkin-Elmer series 200, and the column oven a Perkin-Elmer LC 101. The programmable UV–visible detector was a Perkin-Elmer/Kratos 783. Data processing was carried out on a Multichrom 2 system (Fisons Instruments, Danvers, MA, USA). Extracts were concentrated by evaporation using a Rapidvap System (Labconco, Kansas City, MO, USA). Evaporation was assisted by evacuation with a Maxima C Plus vacuum pump (Fisher Scientific).

2.3. Method A

Syringe-type SPE cartridges containing 100 mg of Dual Zone C_{18} were prepared by treatment with 2 ml of methanol followed by 2 ml of HPLC-grade water. A 0.5-ml serum sample was diluted with 1.0 ml of HPLC-grade water before loading. Loading was carried out either under gravity or by gentle intermittent finger pressure from a syringe attached with an adapter. Loosely-held serum was forcefully expelled after loading by pushing air through the cartridge using the syringe and adapter. The SPE column was washed with 0.5 ml of HPLC-grade water, which was forcefully ejected after washing. Elution into 1000×16 mm test tubes was carried out with 1 ml of methanol again with forceful ejection. After vortex–vacuum evaporation to dryness at 40°C the extract was dissolved in 300 μl of methanol. The resulting methanolic solution was filtered and 10 μl of this solution was injected onto the analytical column.

A linear gradient was formed using HPLC-grade water and acetonitrile starting at 5% acetonitrile and ending in 20 min at 45% acetonitrile. Chromatography was carried out on two 150×4.6 mm, 3-μl Luna C_{18} columns at 60°C. The pump rate was 0.85 ml/min throughout the gradient. The chromatographic signal was obtained by measuring UV absorption at 250 nm.

2.4. Method B

Method B is similar to method A with the following exceptions: (a) 0.5 ml of water was used to dilute the 0.5-ml serum sample. (b) Extraction employed an ordinary C_{18} SPE column rather than a Dual Zone C_{18} column. (c) The gradient was formed between 0.004 *M* sulfuric acid and acetonitrile (8 to 63% acetonitrile in 45 min with a 5-min hold at 63%). (d) The chromatographic signal was measured at 265 nm during the first 31 min and at 240 nm thereafter.

Table 1
Average recoveries and correlation coefficients for six A-group HIV drugs[a]

Drug	Mid-calibration concentration (μg/ml)	Average recovery (%)±SD, $n=20$[b]	Average correlation coefficient (r)	Range of correlation coefficient (r) $n=4$
Zalcitabine	5.516	89.8±11.9	0.9995	0.99882–0.99993
Lamivudine	5.929	96.0±17.7	0.9980	0.99329–0.99997
Stavudine	5.929	93.4±13.2	0.9988	0.99673–0.99952
Didanosine	5.929	92.4±28.8	0.9966	0.98753–0.99997
Zidovudine	5.929	100.3±6.2	0.9992	0.99824–0.99963
Ziagen	5.670	102.7±7.4	0.9993	0.99918–0.99945

[a] Recoveries involved the analysis of 20 different serum matrices spiked at the same level.
[b] For zalcitabine $n=18$. There were two outliers.

Table 2
Average recoveries and correlation coefficients for seven B-group HIV drugs[a]

Drug	Mid-calibration concentration (μg/ml)	Average recovery (%)±SD, $n=20$	Average correlation coefficient (r)	Range of correlation coefficient (r) $n=4$
Nevirapine	5.000	99.0±3.3	0.9997	0.99933–1.00000
Indinavir	5.000	101.1±3.8	0.9997	0.99943–0.99998
Delavirdine	5.000	99.1±3.3	0.9991	0.99853–0.99978
Nelfinavir	5.000	100.0±3.7	0.9998	0.99946–0.99995
Saquinavir	2.500	97.4±3.3	0.9994	0.99880–1.00000
Ritonavir	7.500	101.8±3.2	0.9998	0.99920–1.00000
Efavirenz	5.000	98.5±3.4	0.9998	0.99964–1.00000

[a] Recoveries involved the analysis of 20 different serum matrices spiked at the same level.

3. Results and discussion

Method A was tested by the repeated measurement of serum fortified at a mid-calibration level. Because there was concern that there might be significant variation in the efficiency of extraction of drugs from serum-to-serum for different patients, we decided to use serum from different individuals for each of 20 measurements. For convenience the 20 randomly selected serum samples were divided into four groups of five each. For the first group a three-point linear regression was established using, as standard solutions, fortified pooled serum carried through the extraction process. The five samples of serum from five different individuals were spiked at the mid-calibration level and then analyzed. Recoveries were calculated as percentages of the theoretical, i.e., gravimetric concentration. This procedure was repeated for the remaining three groups of A drugs.

Table 3
Extraction efficiencies for six HIV drugs in group A and for seven HIV drugs in group B

Drug	Extraction efficiency (%), $n=4$	SD, $n=4$
Group A		
Zalcitabine	47.2	22.9
Lamivudine	64.7	21.2
Didanosine	85.5	16.6
Stavudine	85.5	16.2
Zidovudine	98.9	8.5
Ziagen	102.5	12.6
Group B		
Nevirapine	93.9	5.7
Indinavir	92.8	4.3
Delavirdine	88.0	5.8
Nelfinavir	92.6	7.1
Saquinavir	92.9	7.4
Ritonavir	93.1	8.1
Efavirenz	92.7	7.0

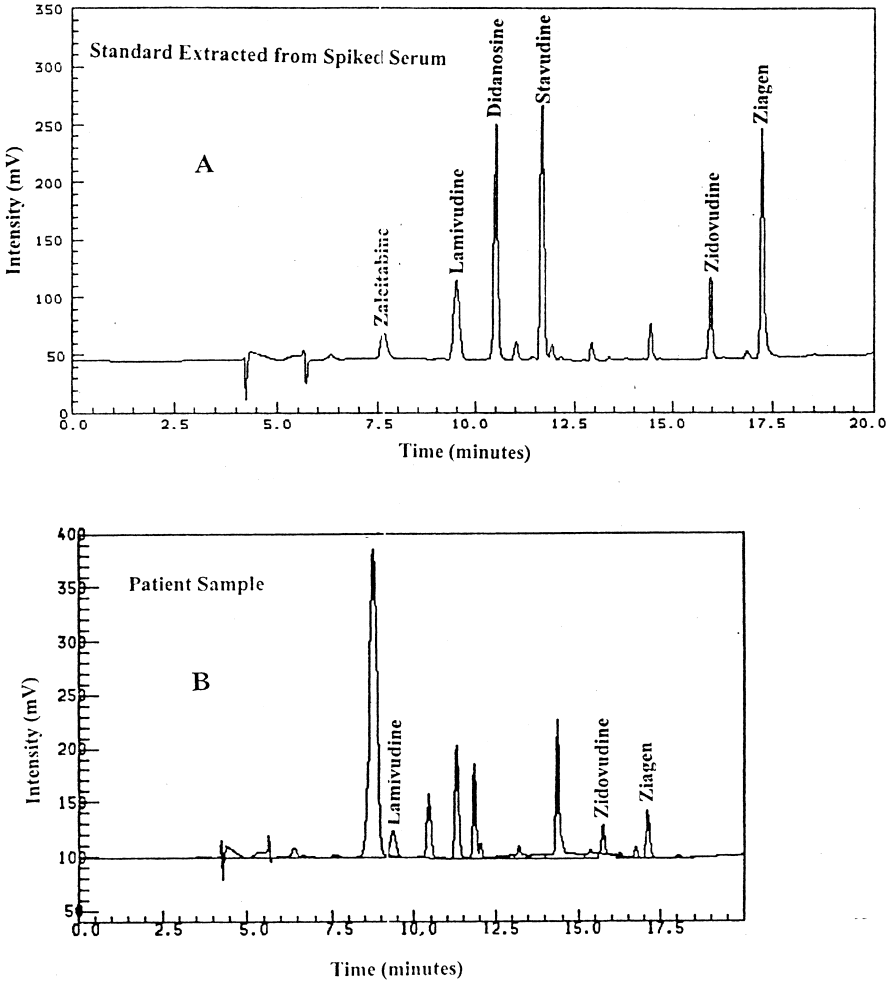

Fig. 1. (A) Chromatogram of the extract of pooled serum spiked at approximately 4.25 μg/ml for each of six A-group drugs. (B) Chromatogram for a patient taking lamivudine, zidovudine and ziagen.

Table 1 shows the concentrations at which measurements were made and the observed recoveries with the corresponding standard deviations (SDs). Table 1 also includes the averages and ranges of the correlation coefficients of four linear regression lines resulting from instrument calibration.

An exactly similar experimental design was carried out for the B-group drugs. The results for the B group are shown in Table 2. The linearity for all drugs appears to be acceptable if not excellent save for one low result for didanosine (0.988). In spite of this SDs for didanosine, and lamivudine are poor (29% and 18%, respectively). We believe that this is due to the fact that all linear regression data were obtained from the same pooled serum, while twenty repetitions of the same spike levels involved sera from 20 different individuals. For the B-group drugs serum-to-serum effects are not troublesome, all SDs in drug recovery being 3 or 4%.

The A group of drugs requires less organic modifier in the mobile phase than the B group. We may conclude that the A group is more polar than the B group. The A group might be expected to be less tightly retained on SPE columns than the B group.

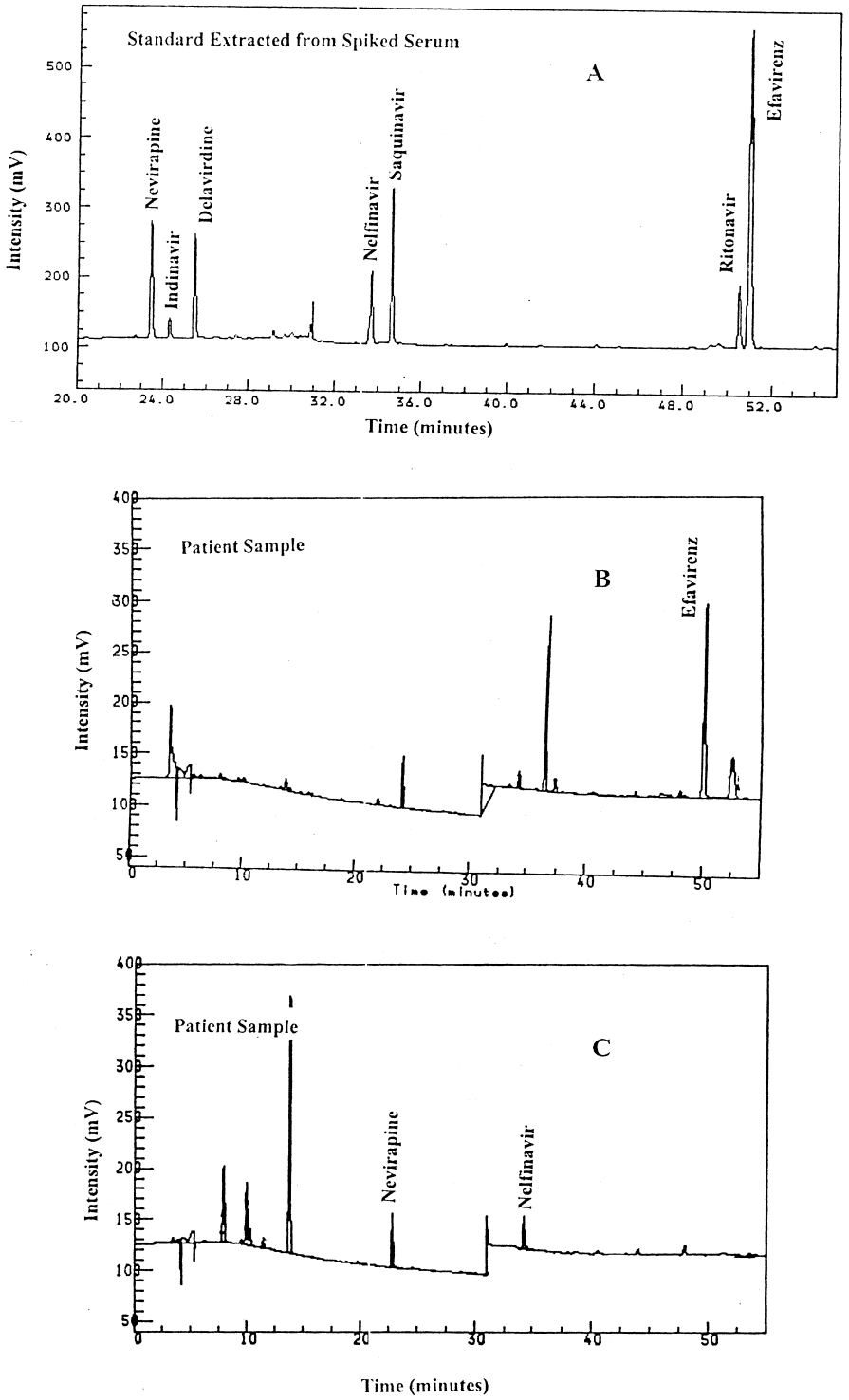

Fig. 2. (A) Chromatogram of the extract of pooled serum spiked with seven B-group drugs. All spiking levels were at 5 μg/ml except for saquinavir, which was at 2.5 μg/ml. (B) Chromatogram for a patient taking efavirenz. (C) Chromatogram for a patient taking nevirapine and nelfinavir.

This is borne out by the observation that doubling the volume of water used in washing the SPE column after loading causes marked loss in peak height for the A group (data not shown). For the A group 35 μl of a standard solution is added to 0.5 ml of serum to produce the levels of the mid-calibration standard as shown in Table 1. We mixed 35 μl of this standard solution with 265 μl of methanol for a total of 300 μl, the same as the final volume of methanol in the extraction process. This solution, which we call the extraction standard, mimics the concentrations which would obtain in the mid-calibration standard if there were 0% losses on extraction. The peak heights of the mid-level calibration standard may be compared directly to those of the extraction standard in order to estimate the extraction efficiency (percent of drug surviving the extraction process). An exactly similar strategy was used for the B group of drugs. The four mid-level standards used all involve extraction from the same pooled serum so that drug-to-drug variation is not obscured by serum-to-serum variation.

Table 3 shows the extraction efficiencies along with their standard deviations for group A and for group B. The generally higher and more consistent extraction efficiencies of group B as compared to group A probably accounts for the generally better analytical results for the former as can be seen by comparing Table 1 with Table 2.

The chromatogram for an extract of serum spiked with the six early-eluting drugs is shown in Fig. 1A together with the chromatogram from a patient (B) treated with lamivudine, zidovudine, and ziagen. In Fig. 2A is shown the chromatogram of an extract of serum spiked with the seven late-eluting drugs together with a patient (B) taking efavirenz and another (C) taking nevirapine and nelfinavir.

The method detection limits (MDLs) for groups A and B are shown in Table 4. The MDLs were determined by the method used by the US Environmental Protection Agency [6]. For both groups the MDLs are sufficiently low to enable results for both methods to be clinically useful.

The upper limit of calibration standards for drugs in group B was 10 μg/ml for all except saquinavir, which because of stronger UV absorbance, was 5 μg/ml. The upper limit of calibration for drugs in group A was approximately 8.5 μg/ml.

Table 4
Method detection limits in serum for six HIV drugs in group A and seven HIV drugs in group B

Drug	MDL (μg/ml)
Group A	
Zalcitabine	0.44
Lamivudine	0.26
Didanosine	0.12
Stavudine	0.040
Zidovudine	0.030
Ziagen	0.075
Group B	
Nevirapine	0.084
Indinavir	0.21
Delavirdine	0.11
Nelfinavir	0.40
Saquinavir	0.10
Ritonavir	0.51
Efavirenz	0.062

4. Conclusions

Two rapid and simple SPE–HPLC methods have been presented which together cover all three pharmaceutical classes of HIV-suppressing drugs. From the analytical standpoint the drugs fall into two classes, viz. early-eluting (group A) and late-eluting (group B) drugs. Separate aliquots of serum and separate injections are required to analyze for both classes of drugs as both the SPE columns and the HPLC gradients are different for the two methods. The method covering early-eluting drugs may be used for the analysis in the therapeutic range of zalcitabine, lamivudine, didanosine, stavudine, zidovudine and ziagen. The late-eluting drug method may be used for the analysis in the therapeutic range of indinavir, nelfinavir, saquinavir, ritonavir, nevirapine, delavirdine and efavirenz.

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