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Trace analysis of impurities in 3′-azido-3′-deoxythymidine by reversed-phase high-performance liquid chromatography and thermospray mass spectrometry

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Abstract

An analytical method has been developed for the detection of trace amounts of impurities in 3′-azido-3′-deoxythymidine referred to herein as AZT (Zidovudine). A sample extract was preconcentrated by normal-phase high-performance liquid chromatography (HPLC) with subsequent on-line reversed-phase HPLC–thermospray mass spectrometry (TSP-MS). During the sample extraction and concentration step, carried out by semipreparative normal-phase chromatography, the preliminary separation of the impurities from the AZT takes place. The organic solvent (dichloroethane–acetonitrile, 40:60) is evaporated from the collected fractions and the compounds are redissolved in a smaller volume of the reversed-phase mobile phases for a further degree of concentration. The collected fractions are then subjected to reversed-phase HPLC–TSP-MS. The influence of acetonitrile concentration and pH on the reversed-phase separation together with the sensitivity of the TSP-MS detection have been examined to maximise detection levels. The 3′-azido-3′-deoxy-5′-O-tritylthymidine, triphenyl methanol and 3′-chloro-3′-deoxythymidine, which are route-indicative impurities formed during the synthesis can be detected in the 50–100 ppb (w/w) range.

1. Introduction

3′-Azido-3′-deoxythymidine (AZT) is the active ingredient in the antiretroviral product manufactured and marketed by the Wellcome Foundation under the trademark Retrovir. AZT has an activity against human immunodeficiency virus (HIV) [1–3] and Retrovir is approved for the treatment of patients with acquired immune deficiency syndrome (AIDS) and AIDS-related complex (ARC) [4–7]. For the study of the plasma and urine concentrations of AZT and its metabolites several reversed-phase HPLC assay methods have been developed [8–10]. The mobile phase compositions applied ranged from 6 to 15% (v/v) acetonitrile in various buffers (phosphate or ammonium acetate with pH 2.7 to 4.5) and have been used to separate the peaks of interest from other endogenous compounds. As our purpose was to develop a method for the detection of route indicative impurities, present at very low concentration (below 0.0005%) in AZT, a single reversed-phase procedure without preconcentration could not be applied. The compounds of interest are more hydrophobic than AZT, and therefore they elute with longer

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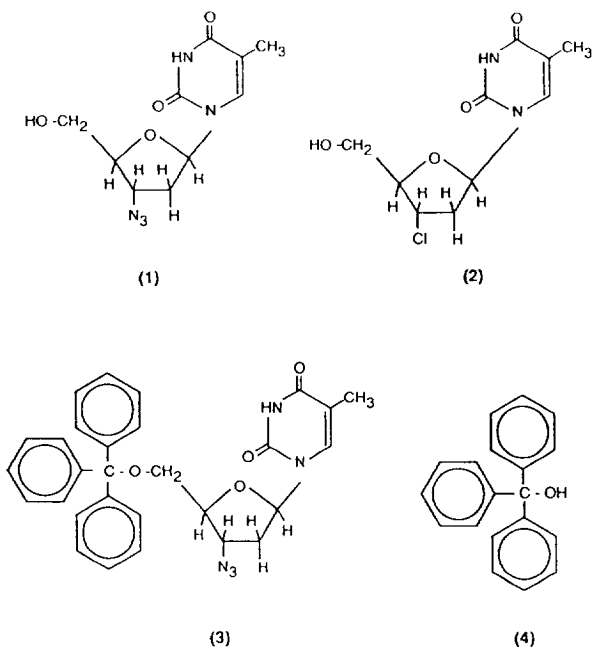

Fig. 1. Chemical structures of the compounds investigated. **1** = 3′-Azido-3′-deoxythymidine (AZT); **2** = 3′-chloro-3′-deoxythymidine (Cl-thymidine); **3** = 3′-azido-3′-deoxy-5′-O-tritylthymidine (trityl-AZT); **4** = triphenyl methanol.

retention times on reversed-phase columns. This is disadvantageous for low level detection. A volatile buffer such as ammonium acetate should be present in the mobile phase for thermospray (TSP) buffer ionisation. The involatile phosphate buffer cannot be used with this technique.

In this paper an analytical method is described by which approximately 10 ppb (w/w) amounts of synthetic route indicative impurities of AZT (Fig. 1) can be detected for purposes of patent protection.

2. Materials and methods

The compounds investigated (**1–4** in Fig. 1) were synthesized by the Wellcome Foundation (UK). Their purity was checked by chemical analysis and chromatography. The acetonitrile was purchased from Romil Chemicals (Loughborough, UK) and was of Super Purity Solvent grade ("190 far UV"). The 1,2-dichloroethane used was HPLC grade and was obtained from Rathburn (Walkerburn, UK). The ammonium acetate and acetic acid were HPLC grade and obtained from Fisons (Loughborough, UK) and BDH (Poole, UK), respectively.

2.1. Sample preparation and preconcentration by normal-phase chromatography

The required amount of AZT either as bulk powder or as a pharmaceutical product (e.g. a tablet or the contents of a capsule) was weighed into a scintillation vial to obtain 600 mg stated amount of AZT; 20 ml of extraction solvent was then added to the vial ("the sample extraction vial"). Another 20 ml of extracting solvent in another scintillation vial was subjected to the same procedures to provide an extraction blank. The extracting solvent was acetonitrile–1,2-dichloroethane (60:40, v/v). This solvent mixture was used as the mobile phase in the normal-phase chromatography. Both vials were placed on a mechanical shaker for 30 min. The 600 mg of AZT are soluble in the 20 ml of extraction solvent but the pharmaceutical product can contain additional insoluble components. After centrifuging both vials for 30 min at 2500 rpm (1500 *g*), the supernatant liquid from the sample extraction vial was removed and transferred into another vial. The contents of both vials, i.e. the sample extraction vial and extraction blank vial, were concentrated under a stream of dry nitrogen. From the sample extraction vial the AZT component continuously precipitated as the solvent volume was decreased. When approximately 4 ml of solvent remained, the sample extraction vial was centrifuged again for 10 min at 2500 rpm (1500 *g*), and the supernatant liquid transferred to another vial. This concentration was continued until approximately 200 μl of solvent remained in both vials. The contents of the sample extraction vial were then filtered using a Millex-HV_{13} 0.45-μm filter (Millipore, Bedford, MA, USA). A 150-μl volume of the extraction blank, followed by 150 μl from the sample extraction were injected into the normal-phase HPLC system.

Two Waters (Division of Millipore, Milford, MA, USA) 510 pumps with automated gradient

controller were used together with a Waters 712 WISP autosampler and Waters 490E programmable multiwavelength detector. The column temperature was maintained at 30°C using an oven unit obtained from Jones Chromatography (Hengoed, UK). The semipreparative Zorbax Sil, 250 × 9.4 mm column was purchased from DuPont (Wilmington, DE, USA) and the silica guard column (10 mm × 4.6 mm) was purchased from Anachem (Luton, UK). The (NP) mobile phase was acetonitrile–1,2-dichloroethane (60:40, v/v), the same as the extracting solvent, with a flow-rate of 2.5 ml/min. The detection was carried out at 265 nm UV with sensitivity 0.05 absorbance unit full scale (AUFS) range. Quantitative evaluations of the chromatograms according to the UV absorbance were made by a Multichrom data acquisition and analysis system (VG Data Systems, Altrincham, UK). The fractions corresponding to the triphenyl methanol, 3′-azido-3′-deoxy-5′-O-tritylthymidine (trityl-AZT) and 3′-chloro-3′-deoxythymidine (Cl-thymidine) were collected manually in scintillation vials. Fractions were collected after injection of the mobile phase as a machine blank (150 μl); the extraction blank (150 μl); followed by the sample extraction (150 μl). The fractions collected were blown down to dryness and redissolved in 200 μl of acetonitrile–water (60:40) (the triphenyl methanol and the trityl-AZT fractions) or acetonitrile–water (8:92) (Cl-thymidine fractions).

2.2. RP-HPLC–mass spectrometry method with TSP ionisation

The redissolved fractions were transferred to autosampler vials, and 100 μl from each vial injected into the HPLC–mass spectrometry (MS) system. A Hewlett-Packard (Waldbronn, Germany) Model 1050 pump unit, ultraviolet detector and the autosampler were used. The reversed-phase column was a Zorbax C_{18} (150 × 4.6 mm) (DuPont). The mobile phase flow-rate was 1.0 ml/min and the column temperature was 30°C. The triphenyl methanol and the trityl-AZT fractions were analyzed by acetonitrile–water (60:40, v/v) with 0.1 *M* ammonium acetate (pH 7), while the Cl-thymidine was analyzed by acetonitrile–water (8:92, v/v) with 0.1 *M* ammonium acetate (pH 3.5; adjusted by concentrated acetic acid). The effluent was monitored by UV at 265 nm and also by a TSP interface to a Fisons (Manchester, UK) VG-TRIO-1000 mass spectrometer. Positive TSP buffer ionisation was applied for the detection of triphenyl methanol detected as a triphenylmethyl cation $(C_6H_5)_3C^+$ (*m/z* 243), and the Cl-thymidine detected as a protonated molecular ion $(M+H)^+$ (*m/z* 261), while the trityl-AZT was detected as a deprotonated molecular ion $(M-H)^-$ *m/z* 508. The TSP conditions were: source temperature 230°C; nozzle temperature was 225°C for the measurements of triphenyl methanol and the trityl-AZT and 235°C for the Cl-thymidine. The repeller voltage was 165 V throughout.

3. Results and discussion

Typical HPLC–UV chromatograms, obtained from normal-phase chromatography of the standard compounds and for the sample extract are shown in Fig. 2. The large peak on the chromatogram of the sample extract (Fig. 2D) corresponds to AZT. The advantage of this normal-phase chromatography is that two of the compounds to be detected are eluted before the AZT peak. The peak of the Cl-thymidine cannot be detected because it is overlapped by the large AZT peak. The typical retention times and the fractions cut are summarized in Table 1. It can be seen that the target impurities are present at a very low level. For example the trityl-AZT peak in the sample extract corresponds to approximately 100 ng of compound injected.

At this stage of the method the most important achievement is the elimination of the AZT from the fractions containing triphenyl methanol and trityl-AZT (fractions B and D, respectively). Unfortunately the fraction containing Cl-thymidine (fraction F) still contains a reasonable amount of AZT. Therefore, this fraction needs to be further separated by the reversed-phase chromatography.

An important criterion of the reversed-phase

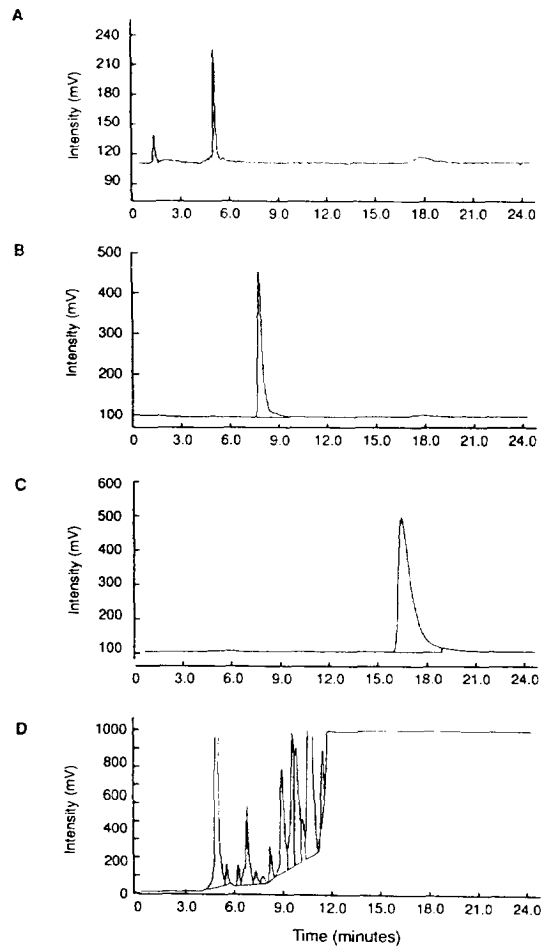

Fig. 2. Typical chromatograms of 1.5 μg injected amount of triphenyl methanol (A), 1.5 μg injected amount of trityl-AZT (B), 1.5 μg Cl-thymidine (C) and the sample extraction of six Retrovir capsules (D). Chromatographic conditions: column: Zorbax Sil 250 × 9.4 mm with silica guard column (10 × 4.6 mm); mobile phase: acetonitrile–1,2-dichloroethane (60:40, v/v); flow-rate: 2.50 ml/min; temperature 30°C; detection: UV at 265 nm.

HPLC–TSP-MS method development is to use mobile phases which give short retention times (preferably less than 10 min) and consequently stronger signals in single ion monitoring without loss of chromatographic resolution. It is also important to have ammonium acetate present to facilitate TSP buffer ionisation. When the mobile phase contains more than 65% acetonitrile it forms an immiscible solution with the aqueous ammonium acetate. Therefore the acetonitrile concentration must be kept lower.

To enhance the detection and sensitivity with the separation of the compounds of interest, their retention behaviour has been investigated on the reversed-phase column by varying the acetonitrile and the ammonium acetate concentrations with the pH. The plots obtained of the log k' values against the acetonitrile concentration at pH 7 for the compounds can be seen in Fig. 3. The triphenyl methanol and the trityl-AZT can be easily separated, even their retention order can be changed. The separation of AZT from the Cl-thymidine is more difficult. The resolution of the two peaks could not be increased by changing the acetonitrile concentration as can be seen in Fig. 3. The separation of the AZT from the Cl-thymidine has also been investigated as a function of mobile phase pH (Fig. 4). It can be seen that by decreasing the mobile phase pH, the retention times of the compounds decreased significantly, due to protonation of the molecules. This allowed a decrease in the acetonitrile concentration, and improved resolution was achieved. Further, the sensitivity of the TSP single ion monitoring at m/z 261 of the Cl-thymidine doubled at a pH below its pK_b.

Fig. 5 shows the retention dependence of triphenyl methanol and trityl-AZT on pH and ammonium acetate concentration by using 60% acetonitrile as organic modifier in the mobile phase. It can be seen that the ammonium acetate concentration shows more influence on the retention of the compounds than the pH. The sensitivity of the TSP-MS for single ion monitoring in the case of triphenyl methanol and trityl-AZT did not increase significantly. Therefore the neutral pH and the commonly used 0.1 M ammonium acetate concentration was selected.

On the basis of the results presented in Figs. 3–5 the typical chromatograms obtained by the optimized mobile phases are shown in Fig. 6.

3.1. Detection limit

The detection limit of the three compounds was determined by injecting progressively less of

Table 1
Typical retention times and the fractions cut from the normal-phase chromatography

Fraction	Time (min)	Compound	Retention time (min)
A	0–4.5	Blank	
B	4.5–5.8	Triphenyl methanol (**4**)	5.0
C	5.8–7.3	Blank	
D	7.3–8.7	Trityl-AZT (**3**)	8.0
E	8.7–13.0	Blank	
F	13.0–17.0	Cl-Thymidine (**2**)	16.0
G	17.0–20.0	AZT (**1**)	17.8

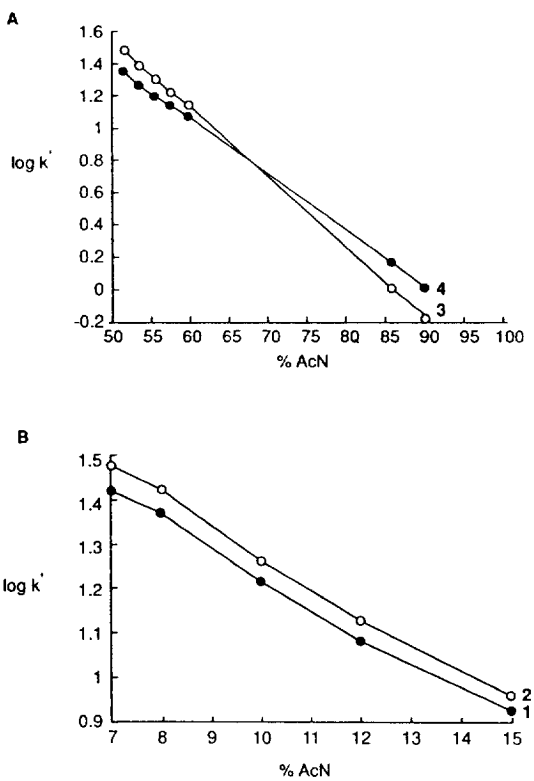

Fig. 3. Plots of the log k' values as a function of the acetonitrile (AcN) concentration of (A) triphenyl methanol (**4**) and trityl-AZT (**3**) and (B) AZT (**1**) and Cl-thymidine (**2**). The mobile was a mixture of acetonitrile and water.

each until the signal-to-noise ratio decreased to 3. By UV detection at 265 nm with a sensitivity range of 0.05 AUFS the detection limits of the triphenyl methanol, trityl-AZT and the Cl-

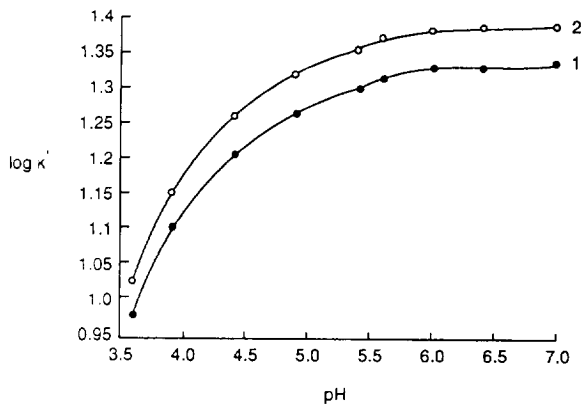

Fig. 4. Plot of the log k' values as a function of mobile phase pH for Cl-thymidine (**2**) and AZT (**1**). The mobile phase was acetonitrile–0.1 *M* ammonium acetate buffer (8:92, v/v).

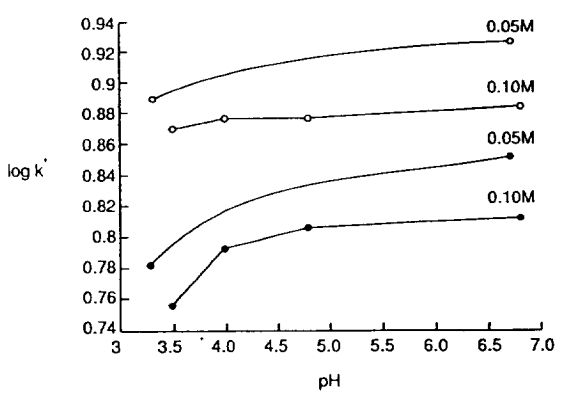

Fig. 5. The retention parameters (log k') of triphenyl methanol (●) and trityl-AZT (○) as a function of the pH and the ammonium acetate concentration of the mobile phase. The mobile phase was acetonitrile–0.05 or 0.10 *M* (as indicated) ammonium acetate solution (60:40, v/v) and pH.

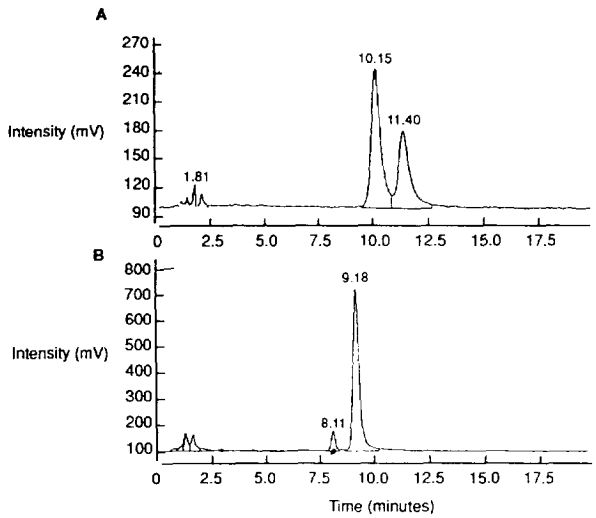

Fig. 6. The optimized separation of (A) Cl-thymidine and AZT and (B) triphenyl methanol and trityl-AZT by reversed-phase chromatography. Column: Zorbax C_{18} 150 × 4.6 mm; flow-rate: 1.0 ml/min; detection: UV at 265 nm. Mobile phases: (A) acetonitrile–0.1 *M* ammonium acetate pH 4 (8:92); (B) acetonitrile–0.1 *M* ammonium acetate pH 7 (60:40, v/v). Injected samples: (A) 210 ng AZT and 16 ng Cl-thymidine, (B) 200 ng triphenyl methanol and 300 ng trityl-AZT.

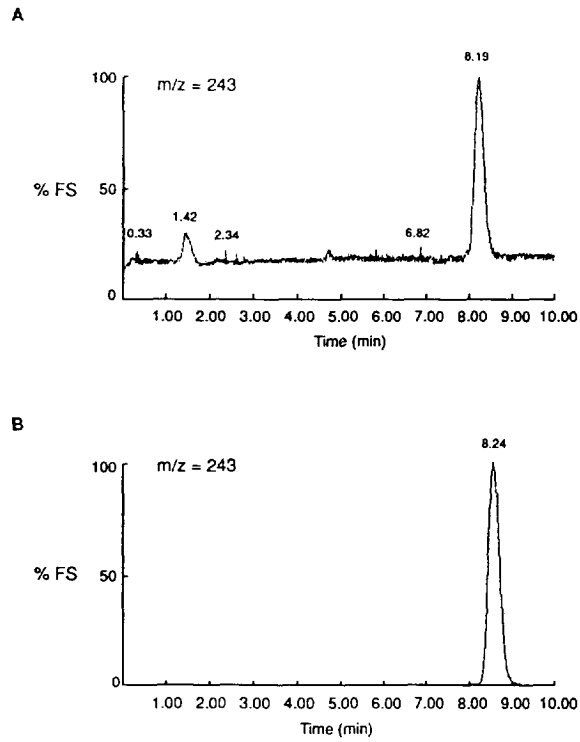

Fig. 7. The reconstructed RP-HPLC–TSP-MS ion chromatograms of 10 ng triphenyl methanol standard (A) and the corresponding sample extraction fraction B (B). HPLC–TSP-MS conditions: Zorbax C_{18} 150 × 4.6 mm column; acetonitrile–water (60:40) with 0.1 *M* ammonium acetate; 1.0 ml/min flow-rate.

thymidine were 10, 1 and 5 ng, respectively. However by TSP-MS single ion monitoring of the peaks the detection limits of the compounds were lower. Figs. 7–9 show the reconstructed ion chromatograms of the triphenyl methanol, trityl-AZT and Cl-thymidine obtained by injecting standards and corresponding sample extraction fractions onto the reversed-phase HPLC–TSP-MS.

3.2. Recovery study

The method presented here is not intended to be quantitative as our purpose was only to detect the synthetic route-indicative impurities. However a recovery study was carried out to reveal the steps where substantial loss of compound could occur and to determine the lowest amount of impurities which can be detected by the method. Substantial loss of impurities can be supposed during the concentration step prior to the normal-phase chromatography. Slow precipitation of AZT during the evaporation stage could co-precipitate the compounds of interest. Further loss (approximately 25%) can be expected by injecting 150 μl from the 200 μl concentrated solutions. The recovery of the compounds from the collected fractions after blowing down completely the acetonitrile–1,2-dichloroethane (60:40, v/v) (mobile phase) and redissolving in the reversed-phase mobile phase was measured for the trityl-AZT and Cl-thymidine. The study was carried out with 625 ng amount of each compound. The recovery of trityl-AZT was 94.8 ± 4.2%, while the recovery of the more polar Cl-thymidine was only 76.0 ± 2.2%.

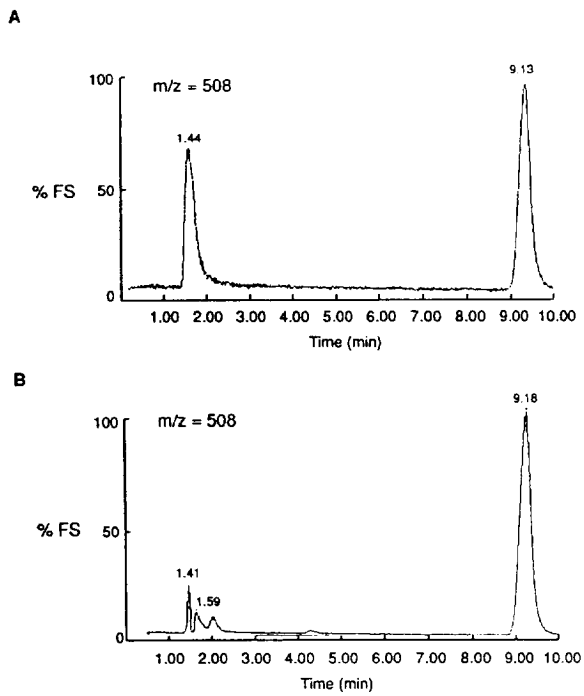

Fig. 8. The reconstructed RP-HPLC–TSP-MS ion chromatograms of 10 ng of trityl-AZT standard (A) and the corresponding sample extraction fraction D (B). The peak at 1.44 min is a pulse caused by injection. Its size is a result of the autoscaling the small sample peak. HPLC conditions as in Fig. 7.

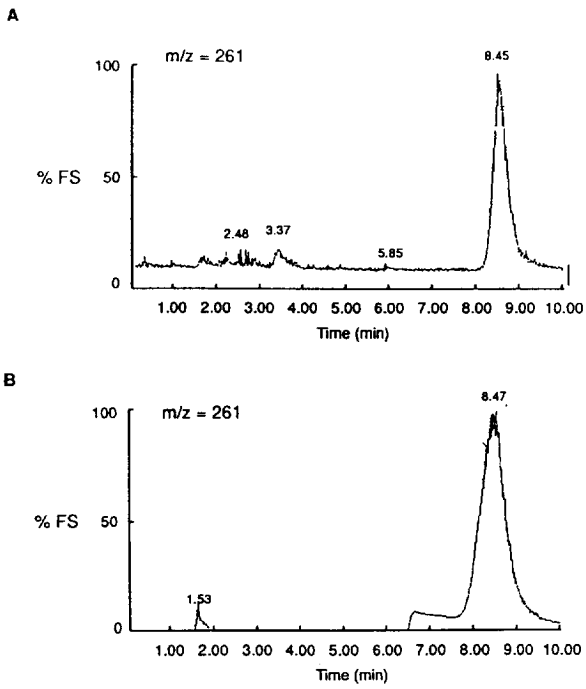

Fig. 9. The reconstructed RP-HPLC–TSP-MS ion chromatograms of 10 ng Cl-thymidine standard (A) and the corresponding sample extraction fraction F (B). The small "hump" before the main peak at 8.47 min is a fragment ion from the large amount of AZT present in this fraction. HPLC–TSP-MS conditions: Zorbax C_{18} 150 × 4.6 mm column; acetonitrile–water (8:92, v/v) with 0.1 *M* ammonium acetate; 1.0 ml/min flow-rate.

The recovery of the triphenyl methanol, trityl-AZT and Cl-thymidine without the presence of AZT was studied by extraction from a solution containing 12.0 ng of each compound. The detected amount of each compound was 1.2 ng triphenyl methanol and 1.0 ng of trityl-AZT. Cl-Thymidine was not detected in this experiment. The efficiency of the recovery by this method is therefore approximately 10% for trityl-AZT and triphenyl methanol and lower for Cl-thymidine. Assuming a significant loss by co-precipitation of the compounds in the precipitating AZT and another 50% loss by injecting of 100 μl from the 200 μl for the HPLC–TSP-MS the method allows us to detect and prove the identity of the route indicative impurities present in the range 50–100 ppb.

In conclusion a very sensitive and selective method has been developed for the detection of route indicative impurities present in AZT formulations. After separating the compounds from the AZT on a normal-phase semipreparative column, the isolated fractions were subjected to reversed-phase TSP-MS analysis. Although it is not a quantitative method it enables detection of the compounds in the 50–100 ppb range.

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