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Short Communication

High-performance liquid chromatographic assay for 2′-deoxy-3′-thiacytidine in human serum

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Abstract

A high-performance liquid chromatographic method for the determination of 2′-deoxy-3′-thiacytidine (3TC), a novel dideoxy-nucleoside analogue, in human serum is described. 3TC was extracted from serum samples using Bond Elut Certify solid-phase extraction cartridges prior to reversed-phase chromatography with UV detection. The method has been shown to be valid over the concentration range 10–5000 ng/ml using a 1-ml sample volume, both before and after heat treatment of the samples at 60°C for 3 h. The method has been automated using a Zymark robot and used in the analysis of serum samples from HIV positive patients.

1. Introduction

3TC (Fig.1), (2*R*, *cis*)-4-amino-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one is a novel dideoxy-nucleoside analogue, which has shown potent *in vitro* activity against a range of lymphotropic and monotropic HIV-1 isolates including zidovudine-resistant strains [1]. To establish its potential therapeutic use for treatment of patients infected with HIV, 3TC is currently being evaluated in phase I/II clinical trials.

An analytical method was required for the determination of 3TC in the low ng/ml range in human serum for monitoring clinical trials and obtaining pharmacokinetic data in man. The potentially hazardous nature of biological samples from HIV positive patients could be reduced by heat treatment [2,3] of the samples at 60°C for 3 h, consequently it was also important to demonstrate stability of 3TC under such conditions. The risk to the analyst of exposure to HIV was further reduced by automation of the method using a Zymark robotic system. The automation required the addition of an internal standard, carbovir (Fig. 1).

2. Experimental

2.1. Chemicals

3TC and carbovir were synthesised by Glaxo Group Research (Greenford, UK). Methanol (HPLC grade) ammonium acetate (Analar grade) and glacial acetic acid (Analar grade) were purchased from Fisons (Loughborough, UK). Acetonitrile (HPLC grade) was purchased

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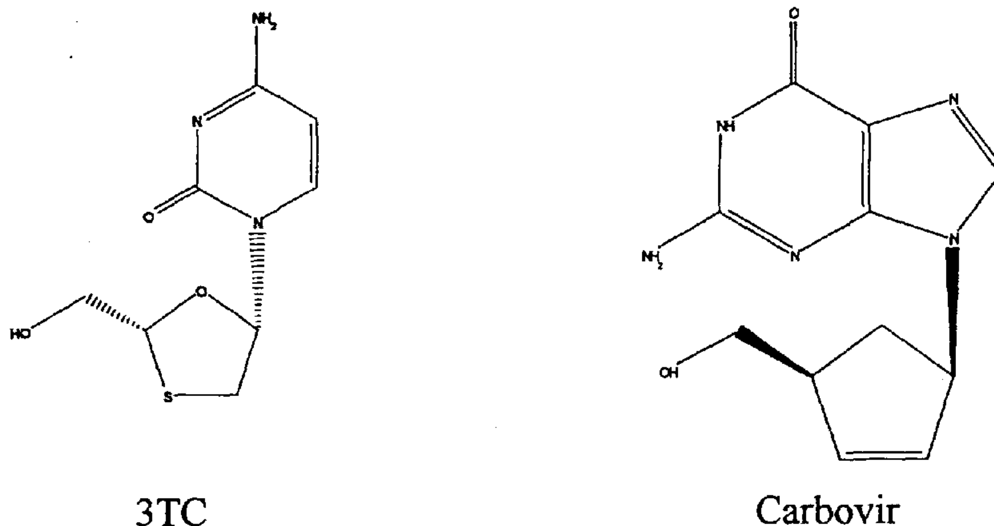

Fig. 1. Structures of 3TC and carbovir.

from Rathburn (Walkerburn, UK). Triethylamine (Sequanal grade) was purchased from Pierce and Warriner (Chester, UK).

2.2. *Instrumentation*

The chromatographic analyses were performed using an HPLC system consisting of a Spectroflow 400 solvent delivery system, a Spectroflow 783 variable wavelength UV detector (both from Kratos, Ramsey, NJ, USA), and a CTO-6A column oven (Shimadzu, Kyoto, Japan). Integration of chromatograms was performed using a Trilab 2000 data system (Trivector, Sandy, UK).

Sample extraction was performed using Bond Elut Certify LRC (130 mg, 10 ml) extraction cartridges (Varian, Harbor City, CA, USA). These cartridges are packed with a mixed-mode phase containing both reversed-phase and cation-exchange elements, which proved to be more selective than either phase used alone. The sample preparation was automated using a Zymate II System (Zymark, Hopkinton, MA, USA) equipped with the following workstations (Pysections): general purpose hand, 2-ml pipet hand, rack, test tube dispenser, weighing-liquid transfer, dilute and dissolve, three master laboratory stations, liquid–solid extraction, evaporation, disposal and LC sipping injection.

2.3. *Preparation of stock solutions of 3TC*

For each validation exercise 3TC was weighed out in duplicate and dissolved in distilled water to give two solutions designated A and B. Dilutions of each stock were made with distilled water as appropriate, including a 10 μg/ml solution. The UV absorbance of each 10 μg/ml dilution was determined at 270 nm to ensure that the drug concentrations of the original solutions were correct. Dilutions of stock A were used in the preparation of the calibration standards and dilutions of stock B were used in the preparation of the validation and quality control samples (QCs).

2.4. *Preparation of calibration standards and quality controls*

Control human serum was spiked with aqueous solutions of 3TC derived from stock A to provide standards in the range 10–5000 ng/ml for the manual assay and 10–2000 ng/ml for the automated assay. Serum QC samples were prepared by spiking control serum with stock B solutions to provide low, medium and high

concentrations of 3TC for the determination of inter-assay performance. Serum was also spiked from stock B to provide samples within the calibration range for the determination of intra-assay performance.

2.5. Preparation of carbovir internal standard solution

A stock solution containing approximately 1 mg/ml of carbovir was prepared in distilled water and this solution was diluted to a concentration of 750 ng/ml in 1% acetic acid. In the automated procedure the application of the sample to the extraction cartridge was achieved by pouring. To allow for any inconsistencies in the automated transfer the internal standard solution described was added to the serum, in stead of the 1% acetic acid used in the manual method.

2.6. Assay procedure

An aliquot of serum (1 ml) was mixed with an equal volume of 1% acetic acid, which, in the case of the automated method, contained 750 ng/ml of the internal standard, carbovir. This mixture was applied to a Certify Bond Elut which had been activated with 2 ml of methanol and 2 ml of 1% acetic acid. The cartridge was then washed sequentially with 2 ml of distilled water, 2 ml of methanol–10% acetic acid (9:1, v/v) and a further 2 ml of distilled water. Finally the cartridge was eluted with 4×0.5 ml of methanol–triethlyamine (9:1, v/v) which was collected into a clean tube and evaporated to dryness at 70°C under a gentle stream of nitrogen. The residue was reconstituted in 200 μl of mobile phase by vortex-mixing, and a 100-μl aliquot was injected onto the HPLC column.

The manual extraction method was performed with a vacuum box operating at 5 mmHg (17 kPa) vacuum, whereas the automated method used 4 mmHg (13 kPa) positive pressure.

A 250×4.6mm I.D. 5 μm BDS Hypersil analytical column (Shandon, Runcorn, UK) , maintained at 40°C, was eluted with a mobile phase of 8% methanol, 1% acetonitrile and 0.1% acetic acid in 0.1 *M* ammonium acetate made up with distilled water at a flow-rate of 1 ml/min. The UV absorption of the column eluant was monitored at a wavelength of 270 nm.

2.7. Intra-assay precision

A set of calibration standards prepared from stock A solutions and six-fold replicates of serum spiked from stock B at the same concentration as six of the calibration standards were analysed, along with the appropriate QC samples, as a single batch using the methods described. The intra-assay variability of the method was determined using the coefficient of variation of replicate assays ($n = 6$) for each of the six selected concentrations on a single occasion.

2.8. Inter-assay precision

On four separate occasions two quality control samples at three concentrations were assayed alongside additional samples and a set of calibration standards. The inter-assay precision was determined as the coefficient of variation for each set of QC samples ($n = 8$). On each occasion samples were added to the batch in order to make it representative in size of a standard analytical batch. In order to accept results for each batch, on each occasion at least four out of the six QC samples, and at least one at each of the three levels, were required to give values within 15% of their nominal concentrations.

2.9. Heat treatment

The inter- and intra-assay precision determinations were performed, using the manual method, on samples which both had, and had not, been subjected to heat treatment. The heat treatment of serum validation samples was performed at 60°C for 3 h in a UT6200 air circulation oven (Heraeus, Germany). The automated validation used serum samples which had not been heat treated.

2.10. Recovery

The absolute recovery of 3TC from serum was determined by comparing the response obtained from aqueous non-extracted solutions of 3TC injected onto the analytical column, with the response obtained from extracted serum samples. Absolute recovery was determined over the range 10–2000 ng/ml, both in the presence and absence of the internal standard, carbovir. Absolute recovery from serum was confirmed using radiolabelled 3TC.

2.11. Specificity

Samples of human serum taken from a number of subjects were tested to determine whether endogenous components would interfere with the analysis. The sulphoxide metabolite identified during animal studies and three other anti-HIV nucleoside analogues (zidovudine, dideoxyinosine and dideoxycytidine) were also examined under these assay conditions for potential interference.

3. Results and discussion

Sample chromatograms of blank serum and a 50 ng 3TC/ml serum standard are presented in Fig. 2. The peak-height response was linear over the calibration range used for both the manual assay, without internal standard, and for the automated assay, using carbovir as the internal standard. In both cases $1/x^2$ weighted linear regression was used in constructing the calibration lines.

A mean calibration line derived from the analysis of standards on five occasions showed close agreement with the same data derived from standards which had been heat treated at 60°C for 3 h prior to analysis. The results indicate that 3TC is stable to heat treatment in serum under the conditions used.

The method displayed good inter- and intra-assay precision and bias over the validated concentration range. Data obtained using the automated, internal standard method are presented in Tables 1 and 2. The corresponding data obtained using the manual, external standard method, before and after heat treatment showed that the manual and automated methods were equivalent and that heat treatment of serum samples had no detrimental effect on assay performance. The limit of quantification for the method was taken to be 10 ng/ml, since this was the lowest calibration standard used and gave a coefficient of variance of 17.4% in the automated method validation.

Extraction recovery data for 3TC from human serum showed that there was no relationship between the concentration of 3TC and absolute recovery, although recoveries appeared to be reduced in the presence of carbovir. The mean recoveries of 3TC, in the presence and absence of carbovir, were 50% and 65%, respectively, however this decrease in absolute recovery did not affect the assay performance.

None of the blank sera analysed showed any endogenous interference in the region of the chromatogram where 3TC elutes. None of the related compounds or potential metabolites examined interfered with the determination of 3TC in serum, because either they were not extracted or did not coelute under the conditions used.

The automated method was used subsequently in the analysis of serum samples from twenty HIV-positive patients during a phase I cross-over study to determine the safety and pharmacokinetics of 3TC, in which the dose was increased in five steps from 0.25 mg/kg to 8 mg/kg. Pharmacokinetic data from this study demonstrated that the mean absolute oral bioavailability was 82%, and that the serum clearance, half-life and volume of distribution were independent of dose [4].

4. Conclusions

An HPLC method for the determination of 3TC in serum has been successfully validated both as a manual (external standard) and an automated (internal standard) process. The method has been shown to be valid over the concentration range 10–5000 ng/ml using a 1-ml

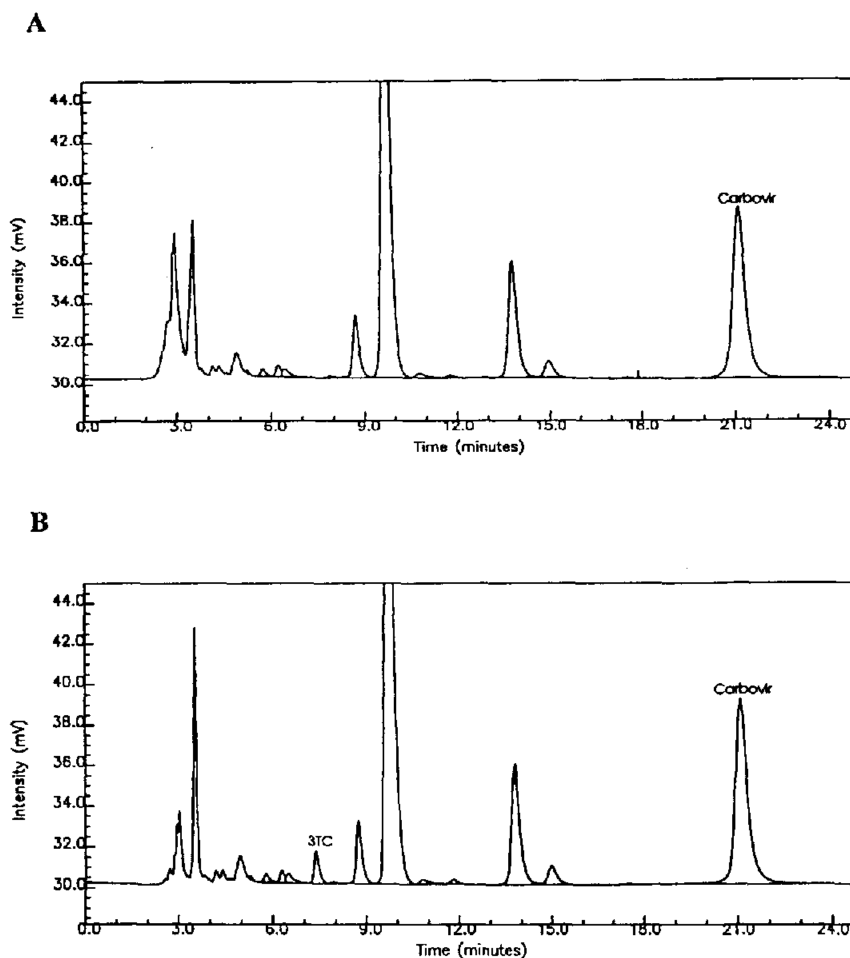

Fig. 2. Sample chromatograms of blank human serum (A) and human serum containing 3TC at 50 ng/ml (B).

Table 1
Intra-assay precision and bias using the automated method

Theoretical ($ng\ ml^{-1}$)	Mean observed ($ng\ ml^{-1}$)	Bias (% error)	Precision (%C.V.)[a]
10.5	10.1	−3.8	17.4
21.0	25.0	+19.0	15.3
49.0	53.0	+8.2	8.3
210	230	+9.5	3.9
490	527	+7.6	4.1
980	1032	+5.3	4.6

[a] C.V. = coefficient of variation.

Table 2
Inter-assay precision and bias using the automated method

Theoretical ($ng\ ml^{-1}$)	Mean observed ($ng\ ml^{-1}$)	Bias (% error)	Precision (%C.V.)[a]
30	31	+3.3	7.4
140	145	+3.6	4.6
403	420	+4.2	5.5

[a] C.V. = coefficient of variation.

sample volume, both before and after heat treatment of the samples at 60°C for 3 h.

Heat treatment of serum samples prior to analysis does not appear to have any detrimental

effect on assay performance. Consequently serum samples from phase I clinical trials involving HIV-positive patients were heat treated and assayed for 3TC using the automated method in order to reduce to a minimum the exposure of laboratory personnel to a potential biohazard.

The analysis of these samples showed the method to be both sensitive and robust. The method is currently being used for the analysis of samples from phase I/II studies as part of the ongoing development programme for 3TC.

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