



ELSEVIER

Journal of Chromatography B, 687 (1996) 457-461

JOURNAL OF
CHROMATOGRAPHY B:
BIOMEDICAL APPLICATIONS

Short communication

High-performance chromatographic assay for the sulphoxide metabolite of 2'-deoxy-3'-thiacytidine in human urine

R.S. Plumb^{a,*}, R.D.M. Gray^a, A.J. Harker^a, S. Taylor^b

^a*Bioanalysis and Drug Metabolism Department, GlaxoWellcome Research and Development, Greenford Road, Greenford, Middlesex, UB6 0HE, UK*

^b*Department of Pharmacology, University of Leeds, Leeds, LS2 9JT, UK*

Received 1 April 1996; revised 21 May 1996; accepted 29 May 1996

Abstract

A high-performance liquid chromatographic method is described for the determination in human urine of GI138870X, the sulphoxide metabolite of a novel dideoxynucleoside analogue, 2'-deoxy-3'-thiacytidine (lamivudine). GI138870X was extracted from human urine using Empore SDB RPS solid-phase extraction disks prior to reversed-phase chromatography with UV detection. The method has shown to be valid over the concentration range 0.5-100 µg/ml using a 0.5-ml sample volume.

Keywords: Lamivudine; 2'-Deoxy-3'-thiacytidine

1. Introduction

Lamivudine, (2*R*,5*S*)-4-amino-1-(2-hydroxymethyl)-1,3-oxathiolan-5-yl)-(1*H*)-pyrimidin-2-one, is a novel dideoxynucleoside analogue which has been granted accelerated approval for the treatment of lymphotropic and monotropic HIV infections in combination with zidovudine. Lamivudine has also been shown to be effective against HIV zidovudine-resistant strains [1]. Lamivudine has been shown to be almost exclusively eliminated from the systemic circulation of the rat and dog into the urine [2]. Data obtained from clinical studies have demonstrated that urinary levels of parent drug only account for about 85% of the absorbed oral dose. As the sulphoxide of lamivudine (GI138870X, compound I) has been

identified as the major metabolite in rat and dog urine after an oral administration, it was considered likely that this sulphoxide metabolite would also be present in the human urine of subjects receiving an oral or intravenous dose. In order to investigate this an analytical method for the determination of GI138870X in human urine in the low µg/ml range was required. The chemical structure of lamivudine and GI138870X are shown below (Fig. 1).

An analytical method has already been developed and validated for the determination of lamivudine in human serum and urine [3,4]. This has been used for monitoring clinical studies and obtaining pharmacokinetic data in man. Due to the polar nature of I it was not possible to use the existing HPLC assay for lamivudine in urine nor the solid-phase extraction (SPE) procedure used in the serum assay for lamivudine, as I was not retained on the cartridge with either assay procedure. Empore disks are a new

*Corresponding author.

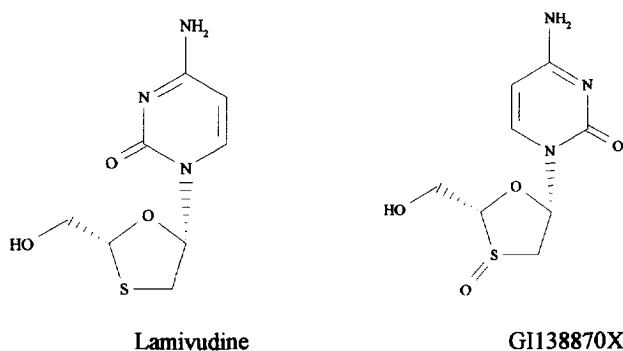


Fig. 1. Structure of lamivudine and GI138870X.

product recently introduced onto the market by 3M [5]. These, unlike traditional solid-phase extraction cartridges have the extraction sorbent impregnated into a thin PTFE membrane into which SPE media is imbedded, rather than a large silica bed, typically of 100 mg. Empore disks are not silica based and thus they do not suffer the chemical limitation of pH found with traditional SPE cartridges and therefore they can be subjected to a wide range of chemical washes.

2. Experimental

2.1. Chemicals

Lamivudine and GI138870X were synthesised by Glaxo Research and Development (Greenford, UK). Methanol (HPLC grade), ammonium acetate (Analar grade) and glacial acetic acid (Analar grade) were purchased from Fisons (Loughborough, UK). Triethylamine (sequanal grade) was purchased from Pierce and Warriner (Chester, UK). Distilled water was produced in house.

2.2. Instrumentation

The chromatographic analyses were performed using a HP1090 integrated, pump, autosampler, column oven and detector (Hewlett-Packard, Palo Alto, CA, USA). Integration was performed using a Multichrom data system Version (VG, Manchester UK). Sample extraction was performed using Empore disk SDBRPS (7 mm/4 ml) extraction car-

tridges (3M, St. Paul, Minneapolis, MN, USA). These cartridges are a mixed-mode phase, based on styrene divinyl benzene, containing both reversed-phase and ion-exchange elements. The sample preparation was carried out on a 50-place vacuum box (Glaxo Bioengineering, Greenford, UK).

2.3. Preparation of stock solutions of I

Two stock solutions were used for the validation exercise; these were designated A and B. The stock solutions were prepared by dissolving duplicate weighings of approximately 10 mg of I authentic standard in 10 ml of distilled water. Dilutions of each stock were made with distilled water as appropriate. Dilutions of stock A were used in the preparation of the calibration line standards and dilutions of stock B were used in the preparation of the validation and quality control samples (QCs). These stock solutions were stored for 1 week at 0–4°C before disposal.

2.4. Preparation of calibration standards and quality controls

Control human urine was spiked with aqueous solutions of I derived from stock A to provide standards in the range 0.5–100 µg/ml. Urine QC samples were prepared by spiking control human urine with stock B solutions to provide low, medium and high concentrations of I for the determination of inter-assay performance. Urine was also spiked with I from stock B to provide samples within the calibration range for the determination of intra-assay

performance. The standards and QCs were stored frozen prior to use.

2.5. Assay procedure

An aliquot of urine (0.5 ml) was mixed with an equal volume of 10% acetic acid. This mixture was applied to a Empore SDB-RPS extraction disk which had been primed with 0.2 ml of methanol and 0.5 ml of 10% acetic acid. The cartridge was then washed sequentially with 0.2 ml of distilled water, and 0.2 ml of methanol–acetic acid (9:1, v/v). The cartridge was finally eluted with 4×0.2 ml of methanol–triethylamine (9:1, v/v). The eluent was collected in a clean tube and evaporated to dryness at 70°C under a stream of nitrogen. The residue was reconstituted in 0.25 ml of distilled water by vortex mixing, and a 20- μ l aliquot was injected onto the HPLC column.

The extraction was performed on a vacuum box operating at 20 mmHg vacuum for the sample addition, washing solvents and eluting solution; no vacuum was used for the priming solutions.

The chromatography was performed on a 250×4.6 mm I.D 5 μ m BDS C18 Hypersil analytical column (Shandon, Runcorn, UK), maintained at 40°C. This was eluted with a mobile phase of 1% methanol and 0.1% acetic acid in 0.1 M ammonium acetate made up with distilled water. The flow-rate was 1 ml/min. After 11 min of the analytical run a pure methanol wash was employed for a duration of 4 min, to elute any strongly retained material; and the column was allowed to re-equilibrate for 10 min with the original mobile phase prior to the next injection. The UV absorption of the column eluent was monitored at a wavelength of 270 nm. The concentration of I was determined by peak-height response using $1/x^2$ linear regression to construct the calibration line.

2.6. Intra-assay precision

A set of calibration standards prepared from stock A 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 method described. The intra-assay variability of the method was determined using the coefficient of variation of

replicate assays ($n=6$) for each of six selected concentrations on a single occasion.

2.7. Inter-assay precision

On six 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 ($n=12$). On each occasion samples were added to the batch in order to make it representative in size of an analytical batch. In order to accept results for each batch, on each occasion at least four out of the six QCs, and at least one at each of the three levels, were required to give values within 15% of their nominal concentrations.

2.8. Recovery

The absolute recovery of GI138870X from urine was determined by comparing the response from aqueous non-extracted solutions of GI138870X injected onto the analytical column, with the response obtained from extracted urine samples. Absolute recovery was determined over the range 0.5–100 μ g/ml; there was no observable variation in recovery across the calibration range.

2.9. Specificity

Samples of human urine from a number of subjects were tested to determine whether endogenous components would interfere with the analysis. The parent compound, two other anti-HIV nucleoside analogues (zidovudine and dideoxycytosine) and the major metabolite of zidovudine (zidovudine glucuronide) were also examined under these assay conditions for potential interference.

3. Results and discussion

GI138870X has been successfully analysed by HPLC following solid-phase extraction with mixed-mode Empore solid-phase extraction disks. The use of the Empore disks has allowed a fast and very effective sample clean-up step which was not pos-

sible with conventional silica-based SPE cartridges. The physical geometry of the cartridges has allowed very small wash and sample elution volumes to be used, which not only saves solvent, but results in faster overall extraction time and a more concentrated sample without compromising analyte recovery. The low elution volume and high sample concentration could allow the direct injection of a proportion of the eluent onto the HPLC system, thus removing the need for an evaporation step. Although in this case the presence of triethylamine in the

eluent precluded the direct injection of the eluent, this procedure has been successfully used by the authors in subsequent analytical methods employing Empore disks.

Sample chromatograms of blank urine and a 10 μg GI138870X/ml urine standard are presented in Fig. 2. The peak-height response was linear over the calibration range of 0.5 to 100 $\mu\text{g}/\text{ml}$. Linear regression with $1/x^2$ was used in construction of the calibration lines. The method displayed good inter- and intra-batch precision and acceptable bias over

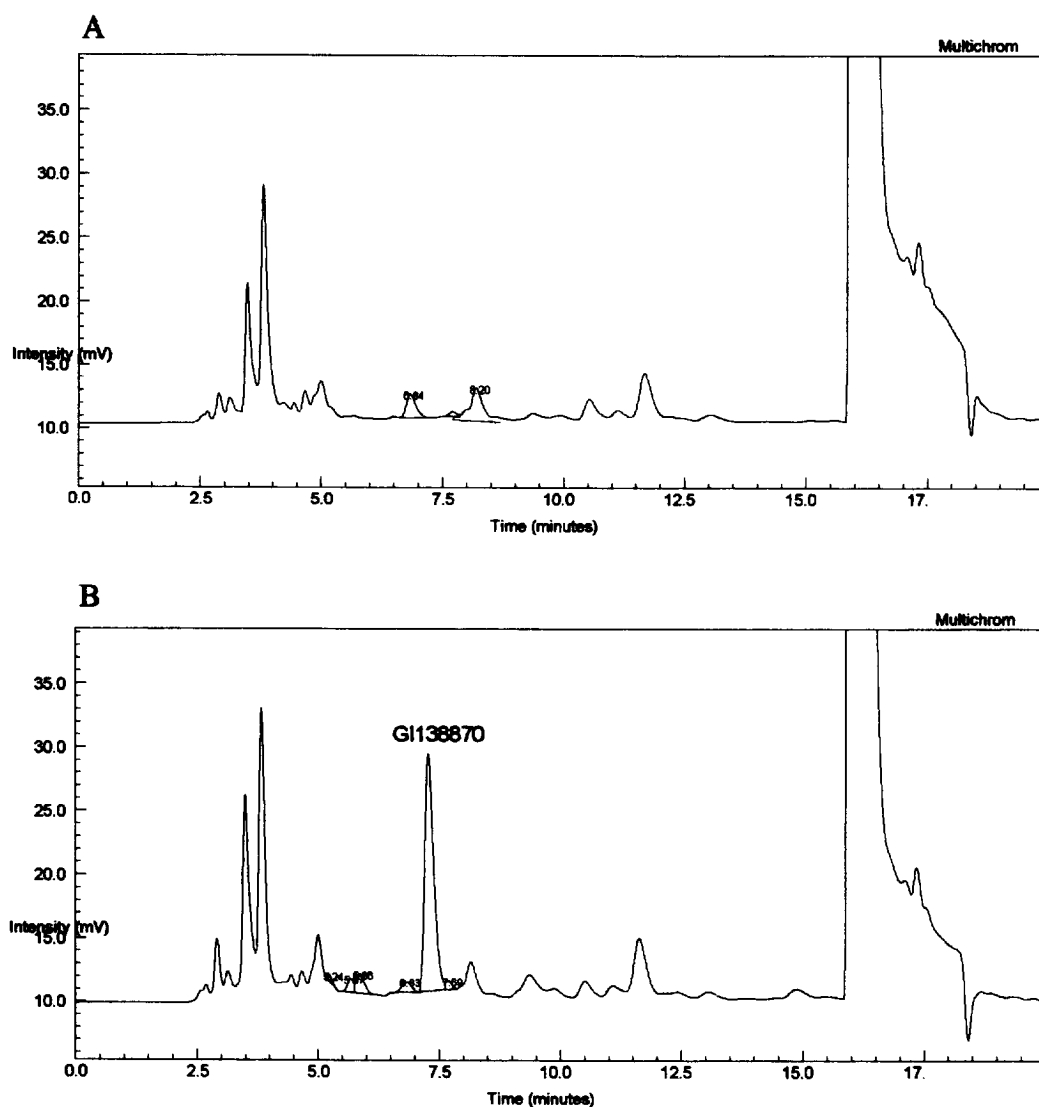


Fig. 2. Sample chromatograms of blank human urine (A) and human urine containing GI138870X at 10 $\mu\text{g}/\text{ml}$ (B).

Table 1
Intra-assay precision and bias ($n=6$)

Theoretical ($\mu\text{g/ml}$)	Mean observed ($\mu\text{g/ml}$)	Bias (% error)	Precision (% C.V.)
0.5	0.56	+3.2	22.4
1	1.04	+4.0	18.2
5	5.40	+8.0	4.7
12.5	11.9	-4.8	7.7
30	26.3	-12.3	8.2
60	52.5	-12.5	6.9

the validated concentration range. Data obtained are presented in Table 1 and Table 2. The limit of quantification for the method was taken to be 0.5 $\mu\text{g/ml}$, since this was the lowest calibration standard used, which gave acceptable accuracy and precision data.

The mean recovery of GI138870X was 65%. None of the blank urines analysed showed any interference in the region of the chromatogram where GI138870X elutes. None of the structurally related compounds examined including lamivudine interfered with the determination of GI138870X in urine, because they were either not extracted or did not coelute under the conditions used.

This method has been successfully used in the analysis of urine samples from twenty-nine patients

Table 2
Inter-assay precision and bias ($n=12$)

Theoretical ($\mu\text{g/ml}$)	Mean observed ($\mu\text{g/ml}$)	Bias (% error)	Precision (% C.V.)
3	2.96	-1.3	10.2
15	15.9	+0.6	6.9
75	79.5	+0.9	5.8

during a study to investigate the pharmacokinetics of a single dose of lamivudine in renally impaired patients and to provide qualitative information regarding any change in renal elimination of the known major sulphoxide metabolite. Data from these patients were compared to a control group with normal renal function.

4. Conclusion

An HPLC method for the determination of I in human urine has been successfully validated. The method has shown to be linear over the concentration range 0.5–100 $\mu\text{g/ml}$ using 0.5-ml sample volume. The method has been shown to be both selective for I and robust. The method has been used for the analysis of samples from clinical studies as part of the ongoing development of lamivudine.

References

- [1] J.A.V. Coates, N. Cammack, H.J. Jenkinson, A.J. Jowett, M.I. Jowett, B.A. Pearson, C.R. Penn, P.L. Rouse, K.C. Viner and J.M. Cameron, *Antimicrob. Agents Chemother.*, 36 (1992) 733.
- [2] Glaxo Research and Development Expert Report on the Preclinical Documentation of Lamivudine.
- [3] A.J. Harker, G.L. Evans, A.E. Hawley and D.M. Morris, *J. Chromatogr. B*, 657 (1994) 227–232.
- [4] D.F. Hagen, G.G. Markell and G.S. Schmitt, *Anal. Chim. Acta*, 236 (1990) 157–164.
- [5] G.C. Mattern, J.B. Louis and J.D. Rosen, *J. Assoc. Off. Anal. Chem.*, 74 (1991) 982.