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Determination of 1-(β-D-arabinofuranosyl)-5-(1-propynyl)-uracil and a metabolite, 5-propynyluracil, in plasma using ASTED (automated sequential trace enrichment of dialysates) combined, on-line, with high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic assay was developed for the determination of the potential antiviral drug 1-(β-D-arabinofuranosyl)-5-(1-propynyl)uracil (I) and a metabolite, 5-propynyluracil (II), in plasma. Plasma samples were mixed with monochloroacetic acid to reduce the effect of protein binding. The mixture was dialysed prior to concentration of analytes on an ODS cartridge followed by isocratic separation on an ODS analytical column using acetonitrile in aqueous ammonium acetate. Detection was by ultraviolet absorption. The quantifiable limit for both I and II is 0.2 μmol/l with a mean inter- and intra-assay precision of 1.3–2.5% (coefficient of variation).

INTRODUCTION

Low concentrations of nucleoside drugs are particularly difficult to monitor in plasma due to their similarity to substances occurring naturally in the matrix. The challenge is therefore to select a sample preparation technique to remove as many similar but unwanted compounds as possible without seriously affecting recovery of the drug for analysis. This paper describes a method for analysis of the nucleoside 1-(β-D-arabinofuranosyl)-5-(1-propynyl)uracil (I), previously referred to as PYaraU, and its metabolite, 5-propynyluracil (II), in human plasma by high performance liquid chromatography (HPLC). The method has also been found to be applicable to plasma from several animal species. Compound I is a potential antiviral drug under development to act against varicella-zoster virus (VZV), the causative agent of chickenpox and shingles [1].

Nucleosides are often thermally unstable, have low volatility and exhibit high polarity giving HPLC something of an advantage over other methods of separation such as gas chromatography which may require a derivatisation step to achieve satisfactory determination. Direct injection of plasma onto the HPLC system produces rapid deterioration of the chromatography due to matrix components. However, several sample pretreatment procedures are available [2]. While the authors have achieved some clean-up success

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with protein precipitation other sample pretreatments have proved to be less than useful. Compound I does not have the advantage of a *cis*-hydroxy configuration for solid-phase extraction (SPE) using phenylboronic acid cartridges as used for pseudoephedrine [3], and no other SPE cartridge studied, for example amino and cyano phases, satisfactorily retained and released both compounds with any reasonable recovery.

The most effective approach achieved was through dialysis to remove the bulk of matrix macromolecules followed by column switching to concentrate and clean up the dialysate for injection onto the HPLC system. A commercially available instrument combining dialysis and column switching for automatic on-line sample preparation was used. The principles of the ASTED (automated sequential trace enrichment of dialysates) and its optimisation for nucleosides have been described [4,5].

EXPERIMENTAL

Chemicals

HPLC-grade acetonitrile and methanol were obtained from Rathburn (Walkerburn, UK). Analytical reagent grade ammonium acetate, glacial acetic acid, monochloroacetic acid (MCA) and sodium chloride and HPLC-grade water were obtained from BDH (Poole, UK). Compound I and its metabolite were obtained from the Wellcome Foundation (Beckenham, UK).

Preparation of standards

Standard solutions of I (10 mmol/l) were prepared in water. Solutions of II were prepared by dissolving in methanol and making up with water (final solution 2.5% methanol). Working, mixed standards (10–100 μmol/l) were obtained by diluting with water. Spiked plasma calibration standards were prepared from the working standards, with a maximum concentration of water of 5%, to cover the range 0.2–50 μmol/l.

High-performance liquid chromatography

A Shimadzu liquid chromatograph (Dyson Instruments, Houghton-le-Spring, UK) consisted of two Model LC7A pumps and a Model SPD-7AV UV detector with an 8-μl flow cell operated at 290 nm. Peak areas were recorded on a Chromatopac C-R4A integrator. The analytical column (150 mm × 4.6 mm I.D.) was packed with 5 μm particle diameter Spherisorb ODS2 (HPLC Technology, Macclesfield, UK).

Isocratic conditions were used to elute I and II with a mobile phase of 4% acetonitrile in 0.004 *M* aqueous ammonium acetate adjusted to pH 5 with acetic acid. A step gradient to 80% acetonitrile at 9 min for a duration of 3 min cleaned the column ready for the next injection.

On-line dialysis and trace enrichment of the dialysate

The sample separation system was a Gilson ASTED module (Anachem, Luton, UK) consisting of a Model 231 autosampling injector, two 401 dilutors equipped with 1-ml syringes and a flat-bed dialyser fitted with a 15 000 molecular mass cut off Cuprophan membrane. The donor cell volume was 370 μl and recipient cell volume 650 μl. A Model 7010 automated six-port Rheodyne valve connected the trace enrichment cartridge (70 mg Prelute ODS, Anachem) either with the recipient channel of the dialyser or with the analytical column of the HPLC system.

ASTED operating conditions

The ASTED was operated in the concurrent mode with injections every 28 min. Dialysis and enrichment parameters were optimised according to procedures discussed in ref. 5.

The plasma sample injected into the donor channel was held static while 950 μl of 2 mmol/l MCA were transported in the pulse mode through the recipient channel to the trace enrichment cartridge. The volume of recipient solution through the dialyser was divided into 1.46 pulses of 650 μl. After each pulse the recipient solution was held static for 2 min 6 s. Upon switching of the six-port valve the enrichment cartridge was eluted for 8 min with the HPLC mobile phase to bring the analytes onto the analytical column. After chromatographic separation the cartridge was washed with 80% (v/v) aqueous acetonitrile,

the recipient cell with 2 mmol/l MCA and the donor cell with 0.86% (v/v) sodium chloride.

Sample preparation

To reduce the effect of protein binding, the ASTED was programmed to automatically mix an aqueous solution of MCA (0.5 mol/l) with the plasma sample prior to dialysis.

Data treatment

Calibration standards were placed at the beginning and end of each analytical run. The mean peak area of each concentration was taken to construct the calibration curve for quantification of plasma samples to allow for the slight deterioration of column performance which infrequently occurred during a run.

RESULTS

The accuracy and precision were determined by analysis of spiked plasma samples. Chromatographic peak areas of analytes were used for calculations. Figs. 1 and 2 show examples of chromatograms of predose and postdose plasma samples from a volunteer dosed with I.

Validation

Precision and accuracy. Within- and between-assay precision and accuracy data over the range 0.2–25 μmol/l are shown in Tables I and II for pre-study validation experiments. Table III shows precision and accuracy data calculated from quality control samples injected during use of the method to support a clinical study.

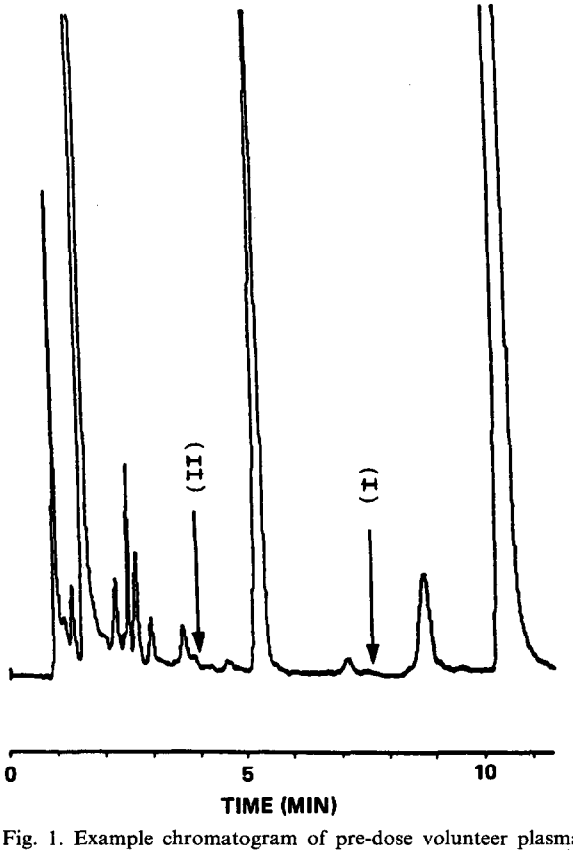

Fig. 1. Example chromatogram of pre-dose volunteer plasma obtained using conditions described in Experimental.

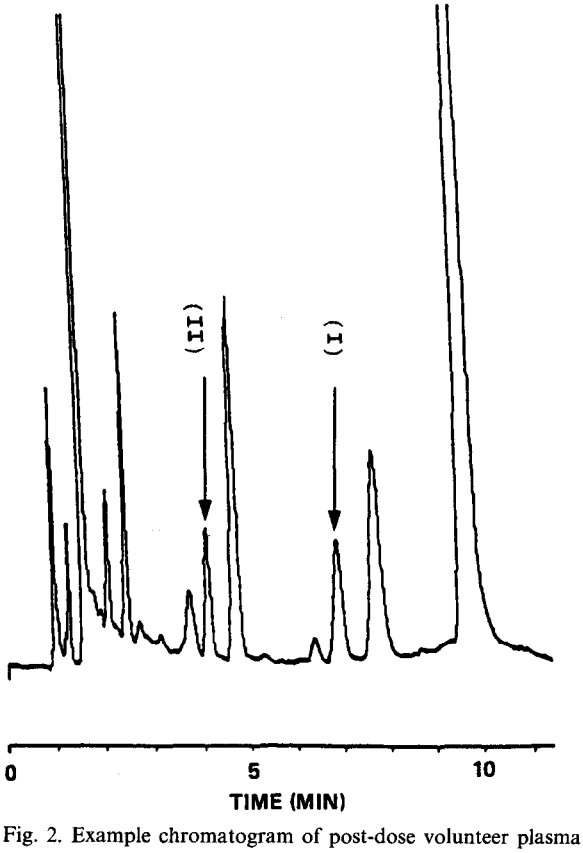

Fig. 2. Example chromatogram of post-dose volunteer plasma containing approximately 1 μmol/l I and II obtained using conditions described in Experimental.

TABLE I

WITHIN-RUN PERFORMANCE OF THE METHOD

n = 6 for each observation.

Concentration (μmol/l)	I		II	
	Accuracy (% bias)	Precision (% C.V.)	Accuracy (% bias)	Precision (% C.V.)
0.2	+0.8	1.2	+1.7	2.1
0.4	−0.3	1.5	−0.7	1.6
1.0	−0.8	4.2	+1.7	1.4
5.0	−1.8	1.1	+1.0	0.7
10.0	+2.3	1.0	+0.9	1.0
25.0	+0.8	0.7	+0.3	0.8
Mean	+0.2	1.6	+0.8	1.3

TABLE II

BETWEEN-RUN PERFORMANCE OF THE METHOD

Data from four runs, six results at each concentration within each run.

Concentration (μmol/l)	I		II	
	Accuracy (% bias)	Precision (% C.V.)	Accuracy (% bias)	Precision (% C.V.)
0.2	+4.4	3.5	+6.3	4.0
0.4	+1.3	1.7	+0.9	3.4
1.0	−0.4	2.7	+0.9	1.5
5.0	0	1.9	+0.3	2.0
10.0	+2.2	1.4	+0.7	1.9
25.0	+0.6	2.4	−0.1	2.2
Mean	+1.4	2.3	+1.5	2.5

TABLE III

BETWEEN-RUN PERFORMANCE OF THE METHOD FROM QC DATA

n = 17.

Concentation (μmol/l)	Precision (% C.V.)	Accuracy (% bias)
I		
2.0	3.23	−1.75
10.0	3.66	−1.32
20.0	2.69	−1.06
II		
2.0	3.51	−1.03
10.0	3.29	+0.16
20.0	1.50	+0.32

Recovery. The relative recovery (by comparison with determination in aqueous solution) of analytes in plasma through the method is shown in Table IV. Proximity of the values to 100% indicates the effectiveness of MCA as a protein binding competition reagent since relative recoveries without MCA were 10–20%.

Limit of quantification. The lower limit of quantification was determined as 0.2 μmol/l for both compounds. The limit was verified in plasma from several subjects to ensure robustness of the method.

Linearity. Calibration curves (six points) show linearity of detector response with analyte concentration over the range 0.2–50 μmol/l: I, slope

TABLE IV

RELATIVE RECOVERY OF ANALYTES

n = 6 for each observation.

Concentration (μmol/l)	Relative recovery (%)	
	I	II
0.2	100.0	104.2
0.4	100.0	104.2
1.0	100.0	104.2
5.0	102.0	104.2
10.0	102.0	104.2
25.0	102.4	105.0
Mean	101.1	104.3

= 20389, S.E. of slope = 42.7, intercept = 173.1, S.E. of intercept = 477.8, correlation coefficient = 0.999982; II, slope = 17619, S.E. of slope = 42.1, intercept = 2465, S.E. of intercept = 471.2, correlation coefficient = 0.99997.

Specificity

Selected clinical plasma samples from volunteers dosed with I were submitted for analysis by HPLC–mass spectrometry which confirmed the identity of analytes present in the plasma. The mass spectrometer was a Finnigan MAT TSQ70 with thermospray source using single ion and selected reaction monitoring. The aerosol temperature was 250°C, vapouriser temperature 100°C and repeller voltage +70 V to +90 V for positive ion and −90 V for negative ion. HPLC conditions were similar to those described previously.

Stability

Aqueous standard solutions of I and II were found to be stable for several months at 4°C but the standard procedure was to prepare fresh stock solutions monthly. Plasma spiked with I showed no evidence of decay when stored at −20°C for seven weeks and less than 4% for II for the same period and temperature.

DISCUSSION

Successful liquid chromatography of nucleoside drugs often consists of manipulating mobile phase conditions to place the peaks due to drugs into very narrow windows between a multitude of peaks from endogenous substances. The lack of robustness caused by such fine tuning can lead to difficulties in the routine analysis of plasma for the provision of pharmacokinetic data. Results of the method described clearly indicate successful removal of many unwanted peaks in addition to macromolecules which cause gross and rapid deterioration of the analytical column. Such improvement in the chromatography provides the opportunity to focus analyte peaks within wide windows of low background interference. The method has been used successfully by the authors [6] for the analysis of I and II in plasma from other species such as mouse, monkey, dog, rat and rabbit and up to 200 μmol/l.

Several thousands of samples have been analysed over many years of usage of the method which has also been transported to contract laboratories.

The analytical method described has been used to support studies to determine the pharmacokinetics of single oral doses of I in healthy volunteers. Plasma profiles for four dose levels are shown in Figs. 3 and 4 and preliminary details of that study are given in ref. 7.

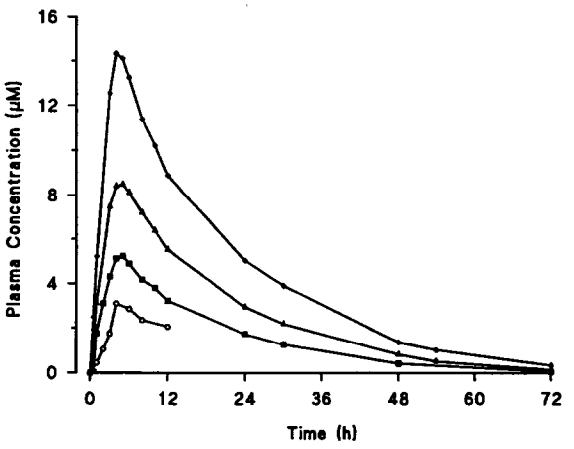

Fig. 3. Mean plasma profiles of I after single oral doses of (○) 50 (n = 2), (■) 100 (n = 9), (▲) 200 (n = 8) and (◆) 400 (n = 8) mg in healthy volunteers.

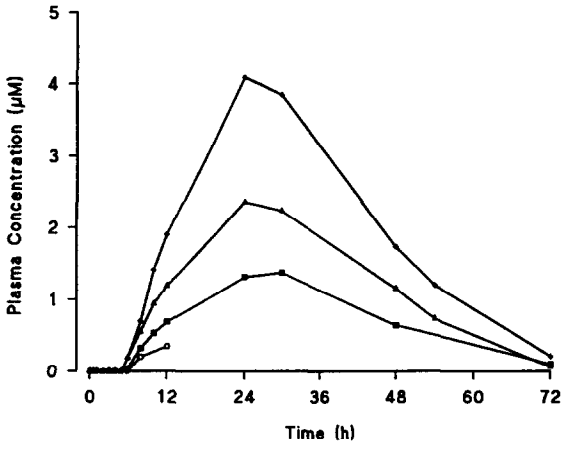

Fig. 4. Mean plasma profiles of II after single oral doses of (○) 50 (n = 2), (■) 100 (n = 9), (▲) 200 (n = 8) and (◆) 400 (n = 8) mg of I in healthy volunteers.

In spite of successful application of the method a combination of ODS packing material in both enrichment cartridge and analytical column does not maximise the selectivity achievable from the column switching aspect of the equipment.

Enrichment cartridge breakthrough profiles for I and II clearly indicate much leaching from the cartridge during the concentration phase leading to absolute recoveries between 10 and 20% and is a consequence of using the low-polarity ODS phase to interact with a polar nucleoside. However, a satisfactory limit of quantification has been achieved and automation of the method by ASTED has produced highly acceptable levels of precision.

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