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Automated sequential trace enrichment of dialysates combined with high-performance liquid chromatography and automated heart-cutting for the determination of the nucleoside 1-(β-D-arabinofuranosyl)-5-(1-propynyl)uracil and its metabolite 5-propynyluracil in urine

J.D.H. Cooper[a,*], C.T.C. Fook Sheung[b], A.R. Buick[b]

[a] *Clinical Innovations Ltd., Building 45, Stoneleigh Deer Park, Stareton, Kenilworth, Warwickshire CV8 2LY, UK*
[b] *Department of Bioanalysis and Drug Metabolism, Wellcome Research Laboratories, Langley Court, South Eden Park Road, Beckenham, Kent BR3 3BS, UK*

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Abstract

The use of a new configuration to control the automated sequential trace enrichment of dialysate (ASTED) system has been used to estimate 1-(β-D-arabinofuranosyl)-5-(1-propynyl)uracil and its metabolite 5-propynyluracil in urine. The system employs "heart-cutting" as a means to improve the efficiency of sample preparation and reduce analysis time. Using this technique the mean within- and between-run imprecision (coefficient of variation) at three different urine analyte concentrations was found to be 1.6 and 3.6% and 1.7 and 3.3% for 5-propynyluracil and 1-(β-D-arabinofuranosyl)-5-(1-propynyl)uracil, respectively.

1. Introduction

Previous publications [1–3] involving the ASTED (automated sequential trace enrichment of dialysates) system have invariably utilised the combination of dialysis and trace enrichment to prepare samples in a completely automated manner. Although satisfactory for a number of applications operational limitations for the combined processes have been exposed. In order to extend the analytical capabilities of dialysis combined with trace enrichment a new ASTED process incorporating "heart-cutting" (process 4) and its application to the estimation of the nucleoside (arabinofuranosyl)-5-(1-propynyl)-uracil (I) and its metabolite, 5-propynyluracil (II), in plasma has been reported [4].

In general the preparation of urine samples presents some analytical problems. This type of matrix is relatively more complex than plasma or serum and much more extraneous interfering compounds may be observed chromatographically especially when UV detection is applied.

* Corresponding author.

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This report describes the application of ASTED process 4 to the analysis of I and II in urine samples. Separation of the analytes in the prepared sample extract was performed using either isocratic- or gradient-elution HPLC and UV detection.

2. Experimental

2.1. Instrumentation

Unless otherwise stated all of the equipment in the HPLC and ASTED units was obtained from Anachem (Luton, UK).

HPLC

The gradient HPLC system consisted of Models 306 and 307/5SC pumps, an ABI 783A variable UV detector and a Rheodyne 7010 injection valve fitted on the ASTED unit. Control of the HPLC system (either in isocratic or gradient mode), integration of chromatographic peaks and communication with the ASTED system (via Gilson Medical Electronics GSIOC) was done with a 714 system controller (Amstrad PC2286/40 with hard disc, EGA graphic card, mouse, MS DOS 3.1 and Windows software).

Sample preparation (ASTED) unit

The software and hardware modifications to an ASTED unit to enable "heart-cutting" have been described previously [4]. The modified ASTED unit basically consisted of a 231 autosampling injector; two 401 dilutors fitted with 1-ml syringes (controlling sample pre-treatment on the donor side and dialysate flow on the recipient side of the dialyser); a universal valve-switching module to isolate the dialyser unit from the trace enrichment device to allow "heart-cutting" routines; a Kel F dialyser unit with a 370-μl donor volume fitted with a 15-kDa molecular mass cut-off Cuprophan membrane, and a stainless-steel trace enrichment cartridge (Prelute, Anachem) packed with 70 mg of 10-μm Hypersil ODS (Shandon Southern Products, Runcorn, UK) to replace the loop on the Rheodyne 7010 injection valve. Control of the ASTED operations was made using Process 4 V1.01 software (Clinical Innovations, Kenilworth, UK).

2.2. Reagents

Unless otherwise stated, all chemicals were analytical grade obtained from BDH Chemicals (Poole, UK). HPLC grade water, prepared using an Elgastat UHQ system (Elga, High Wycombe, UK), was used for all reagent preparations. Diammonium hydrogen orthophosphate (DAHP) buffer (100 mmol/l, pH 7.0) and ammonium acetate solution (500 mmol/l, pH 7.0) were prepared. Water was dispensed by Gilson 401 dilutors to purge both the donor and recipient channels and connections of the dialyser unit.

Standard preparations

Compounds I and II were obtained from Wellcome Research Laboratories, Beckenham, UK. A 10 mmol/l stock solution of I in water and a 2.5 mmol/l stock solution of II in 2.5% methanol in water were prepared. Working standards (1000 and 100 μmol/l), each containing both compounds, were prepared by diluting the stock solution in water. Calibration standards (ranging from 2.5 to 500 μmol/l) were prepared by supplementing drug free urine with the working standards. Supplemented urine samples were stored at −20°C if not analysed on the day of preparation.

2.3. Chromatographic conditions

Both isocratic- and gradient-elution mobile phases (degassed before use) were used to optimise the chromatography conditions. The isocratic mobile phase consisted of tetrahydrofuran–DAHP–water (1.3:0.01:98.69, v/v/v) pumped at a flow-rate of 1.4 ml/min. The gradient mobile phase consisted of solvent A tetrahydrofuran–DAHP–water (1.0:0.01:98.99, v/v/v), and solvent B tetrahydrofuran–DAHP–water (15:0.01:84.99, v/v/v). The gradient conditions were at $t = 0$ min: 0%B; at $t = 10$ min: 0%B; at $t = 10.05$ min: 100%B; at $t = 10.50$: 100%B; at

$t = 10.55$: 0%B; at $t = 15$ min the initial conditions are restored and a new cycle can be started. A flow-rate of 1.4 ml/min was employed under all conditions.

The HPLC column (150 × 4.6 mm I.D.) was packed with 5 μm particles of Kromasil ODS (Technicol, Stockport, UK). No guard column was employed. The analytical column was heated to 33°C in a Kariba column heater (Jones Chromatography, Hengoed, Wales, UK). The detector was set at a wavelength of 290 nm and 0.05 AUFS.

2.4. Sample preparation

Sample preparation was carried out in a completely automated manner using the ASTED process 4 system and optimised as follows using gradient elution conditions:

- 250 μl of urine was mixed with 250 μl of 500 mmol/l ammonium acetate solution and 450 μl of this mixture was delivered to the donor channel of the dialyser.
- 800 μl of recipient solvent moved through to the Prelute cartridge in a 4-min time period.
- following enrichment, 150 μl of water was passed through the Prelute cartridge via the 401 dilutor attached to the needle injection unit of the ASTED system.
- the Rheodyne 7010 valve was then switched to "inject" for 100 s and then returned to the "load" position.
- 200 μl of 80% acetonitrile was aspirated from a reagent vial and dispensed through the Prelute cartridge via the injection port. This was followed by 1.0 ml of water using the same 401 dilutor.
- the system was then purged and the Prelute cartridge regenerated for the next sample.

2.5. Quantification

Calibration standards were situated at the beginning and end of each analytical run. A linear regression analysis was performed for the mean peak areas and concentrations of both sets of standards. This was used to calculate test analyte concentrations.

2.6. Quality control

Drug free urine samples (obtained from healthy volunteers) were supplemented with 4, 40 and 400 μmol/l of I and II. These were aliquoted and stored at −20°C. Each level was analysed three times within an analytical batch of samples.

3. Results

3.1. Optimisation of sample preparation and chromatography conditions

Initial chromatographic conditions were established using gradient elution with the sample preparation conditions described. Fig. 1a shows the chromatogram obtained using this method to analyse a urine sample supplemented with a 50 μmol/l of compounds I and II. No chromatographic interferences were observed in the analysis of pre-dose samples obtained from 30 different volunteers during numerous studies of this drug (Fig. 1b). Fig. 2 shows the chromatograms obtained with and without "heart-cutting" and using isocratic mobile phase conditions. These results were obtained when the Prelute cartridge remained in-line with the mobile phase for 20 s (Fig. 2a) or 100 s (Fig. 2b). For both these injections the mobile phase flow-rate was 0.5 ml/min at the start of injection; it was returned to 1.4 ml/min after 100 s to maintain reproducible analyte retention times between the two injections.

3.2. Assay performance

Data on assay performance was obtained using the ASTED process 4 procedure described in conjunction with the gradient elution mobile phases.

Linearity and sensitivity

Peak areas varied linearly over the analytical range employed for both I and II. The sensitivity of the assay was established by analysing 15 drug free urines after standardising the procedure

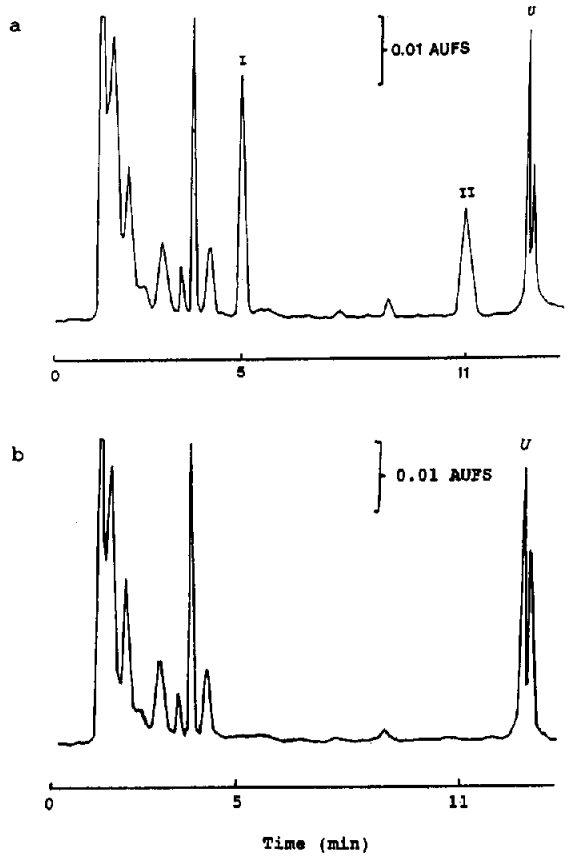

Fig. 1. Chromatograms of a urine sample with (a) and without (b) supplementing with 50 μmol/l of I and II respectively and using the gradient chromatography conditions described. The peaks listed as U are those derived from the stepwise gradient.

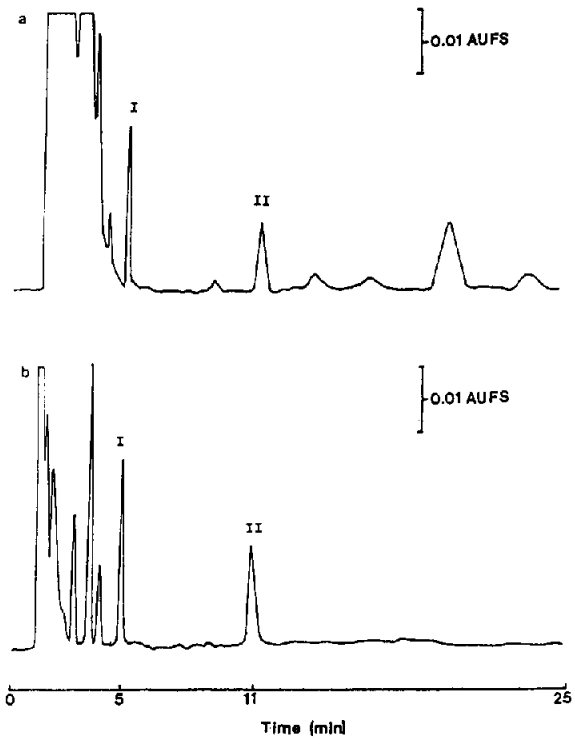

Fig. 2. Chromatogram of a urine sample supplemented with 50 μmol/l of I and II without (a) and with (b) heart-cutting.

with the urine calibrants. The quantification limit detection limit was set at the lowest standard concentration on the calibration curve.

Imprecision

The within-run coefficient of variation (C.V.) was estimated by assaying the quality control samples 15 times in the same analytical run. The between-run C.V. was obtained by estimating the same control samples 15 times on different days. The results are shown in Table 1.

Efficiency of the system

The efficiency of the system was determined by substituting the dialysis/Prelute components with a standard 20-μl loop on the 7010 Rheodyne injection valve and injecting an aqueous working standard solution of I and II onto the HPLC column. The peak areas obtained were compared with those obtained after analysing the same working standard solution using the ASTED process 4 system. The absolute recovery of the analytes was calculated on the basis of the volume of the donor side of the dialyser and was found to be 20.1% and 19.5% for I and II, respectively.

Matrix effects

The relative recovery of the method was found to be 101% ± 2.1% ($n = 10$) by analysing a 200 μmol/l aqueous solution of I and II using the method described. To test for matrix differences between specimens, 10 drug free (pre-dose) urine samples from the volunteers participating in this study were supplemented with 200 μmol/l of I and II and analysed. The mean analytical

Table 1
The within- and between-run imprecision of the method described ($n = 15$ for both within- and between-run estimates)

Analyte	Supplemented concentration (μmol/l)	Estimated mean concentration (μmol/l)	Estimated C.V. %	Bias (%)
Within-run imprecision				
I	4.1	3.9	2.8	−4.6
	41.0	41.3	1.1	0.8
	409.6	399.4	1.1	−2.5
II	4.0	3.9	2.8	−4.0
	40.4	40.2	0.9	−0.1
	402.7	398.7	1.0	−1.0
Between-run imprecision				
I	4.0	4.0	4.7	0.0
	40.0	39.8	2.8	−0.5
	400.0	407.2	2.5	1.8
II	4.0	4.0	5.1	0.0
	40.0	39.8	3.0	−0.4
	399.5	391.4	2.7	−2.0

recovery was found to be $99.6 \pm 1.5\%$ and $97.3 \pm 2.0\%$ for I and II respectively.

4. Discussion

To achieve robust analytical chromatographic methods that are selective for the analytes under investigation depends not only on the use of efficient columns but also in the mode of detection and the efficiency of sample preparation. Care must be taken when analysing samples with complex matrices such as urine. UV detection is not the most ideal mode of detection for urinary extracts and consequently much depends on the efficiency of sample preparation and on the chromatographic conditions to obtain reliable analytical results.

4.1. Sample preparation and chromatography

Initial experiments to prepare urine samples for the chromatographic separation of I and II using solid-phase extraction were not successful due to the high polarity of the analytes under investigation. It could be argued that the use of membranes is not an essential requirement in the preparation of samples such as urines, but the results obtained using on-line dialysis and trace enrichment were more encouraging than those obtained using solid-phase extraction even when using reversed-phase C_{18} enrichment columns. However, the use of membranes is beneficial when analysing urines, protecting the column from particulate matter and, in certain pathological circumstances, from high-molecular-mass contaminants such as proteins.

The use of the ASTED system process 4 for the analysis of I and II in plasma has previously been shown to extend the analytical capabilities of the present commercial unit [4]. Its use in the preparation of urine samples also proved to be beneficial, although when first developing the procedure described in this paper it was considered better to use a stepwise elution gradient in combination with process 4. This avoided the potential chromatographic interference from late eluting peaks, a problem often encountered when analysing complex samples such as urine. A reduction of the time that the Prelute cartridge is in-line with the HPLC mobile phase can effectively perform "heart-cutting", removing unwanted late eluting peaks, reducing the chromatography run-time and thus directly affecting the mode of chromatography adopted. This was demonstrated by using isocratic chromatographic

conditions (Fig. 2) instead of gradient elution (Fig. 1). The net effect of this approach is to reduce the analysis time and increase sample throughput. These beneficial effects could only be accomplished when an HPLC column with a high silica surface area and a low ionic strength buffer in the mobile phase were used. High buffer concentrations in the mobile phase imparted salting-out effects on most of the interfering compounds observed in the chromatogram. Reducing the buffer concentration reduced the column capacities for the interfering peaks without affecting the analytes of interest and by virtue of the wash routine in process 4 a substantial amount of the more polar chromatographic interferences could be eliminated. Further work is now in progress to assess the robustness of the technique when using the system in this mode of operation. As discussed in a previous publication [4] the variables that control the "heart-cutting" routine must be thoroughly examined before optimal assay conditions are obtained.

4.2. Sample preparation and matrix effects

Early reports using the ASTED system [5,6] recognised the influence of different matrices on the diffusion rates of analytes across a semi-permeable membrane. This is of particular importance when dealing with serum samples and when analyte protein binding occurs. Theoretically this should be less of a problem when dealing with urine samples where the matrix does not usually contain high-molecular-mass contaminants. However initial investigations using direct sampling of the urine calibrants showed the method to be non-linear. The inclusion of an automatic pre-dilution of the samples with ammonium acetate solution prior to dialysis eliminated this effect and the technique was then shown to be linear. The reason for this phenomenon is uncertain but it could be due to either variation in ion concentrations between samples affecting the rates of diffusion or to the breakthrough volume of the analytes for the reversed-phase material in the Prelute cartridge. Further evidence to support that elimination of matrix effects had occurred was demonstrated by comparing the recovery of analytes from drug-free urine samples from 10 different volunteers.

4.3. Assay performance

As with the analysis of these compounds in plasma [4] the absolute recovery of the drugs appears low (approximately 20%). The same arguments that were applied to the analysis of plasma samples equally apply to this technique. The reduced recoveries are due to the high polarity of the analytes under investigation resulting in a low column capacity factor on reversed-phase material. This is particularly the case for compound II which has an estimated breakthrough volume (in water) of approximately 1 ml on the Prelute cartridge used in this assay. Other trace enrichment materials may improve this situation and some experiments conducted with a re-configured ASTED system such that minimal pressure is applied across the dialysis membrane gave a two-fold higher recovery than that observed for a conventional ASTED system using similar enrichment volumes and flow-rates. Further work is underway to examine the potential of this ASTED setup. However, at present the analysis of urines is a difficult task; one could argue that some loss of recovery may be justifiable when the sample preparation technique employed minimised the occurrence of interferences and demonstrated acceptable assay performance (Table 1).

This technique provides a rapid approach to the pharmacokinetic examination of compound I and its metabolite II. Furthermore a new generation of ASTED systems under development at Gilson Medical Electronics will encompass the tasks detailed in this report.

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