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Manual and automated determination of 1-β-D-arabinofuranosyl-*E*-5-(2-bromovinyl)uracil and its metabolite (*E*)-5-(2-bromovinyl)uracil in urine

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Abstract

This paper describes the determination of 1-β-D-arabinofuranosyl-*E*-5-(2-bromovinyl)uracil and its metabolite (*E*)-5-(2-bromovinyl)uracil in urine. The method involves sample clean-up by liquid–liquid extraction with ethyl acetate followed by high-performance liquid chromatographic (HPLC) analysis. The sample preparation may be performed either manually or automatically using a Zymark Py-robotic system. The chloro analog of the parent compound, CV-araU, is used as the internal standard. As low as 0.1 μg of analyte per ml of urine can be measured. This sensitivity is adequate for pharmacokinetic studies but could be improved quite easily if necessary.

1. Introduction

Nucleosides and nucleoside analogs have shown specific and potent activity against various viruses [1,2]. One such compound is 1-β-D-arabinofuranosyl-*E*-5-(2-bromovinyl)uracil, BV-araU, which was synthesized by Sakata et al. [3] in 1980. BV-araU has been shown to be one of the most potent and selective inhibitors of the replication of HSV-1 [4–6], *Varicella zoster* virus [7–9], and Epstein–Barr virus [10]. BV-araU is currently being evaluated for clinical use.

It has been reported that in humans approximately 46% of the administered dose of BV-araU was excreted as parent compound in urine over a 24-h period [11]. In order to support clinical studies we developed an assay to determine BV-araU in urine. Earlier, we reported that in man BV-araU undergoes glycosidic cleavage to form (*E*)-5-(2-bromovinyl)uracil, BV-uracil [12]. The strategy therefore was to develop a method to determine not only the parent drug but also the metabolite. There is no method reported in the literature that could be used to assay both parent drug and metabolite in clinical urine samples. One method reported an assay for BV-uracil as a metabolite of a related compound, (*E*)-5-(2-bromovinyl)-2-deoxyuridine (BVdUrD) in plasma but not in urine [13]. Likewise, an assay was described for BV-uracil as a metabolite of BVdUrD in urine [14] but this method lacks the sensitivity required to assay the lower concentrations of BV-uracil as a metabolite of BV-araU in clinical samples. The sample preparation as described in this report is similar to the methodology for BVdUrD reported in

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Ref. [15] and was extended to the measurement of the metabolite BV-uracil at low concentrations. The method can measure as low as 0.10 μg/ml of each analyte per ml of urine. This sensitivity was adequate for assaying samples from Phase I and Phase II clinical studies. The sensitivity of the method can be readily improved by: (a) injecting a larger portion of the reconstituted sample, (b) reconstituting the sample residue in a smaller volume of mobile phase, or (c) assaying a bigger sample size. This method has enhanced precision due to the inclusion of an internal standard, CV-araU, which is the chloro-analog of the parent compound.

In order to conveniently handle the thousands of clinical samples, the sample extraction was automated using a Zymark Py-robot. All the results in this report, except where otherwise indicated, have been obtained using the robot.

2. Experimental

2.1. Materials

BV-araU, BV-uracil, and CV-araU were obtained from Yamasa (Choshi, Japan). Double-distilled water or purified water, obtained by passing it through a Milli-Q reagent water system (Millipore, Bedford, MA, USA), was used. Ammonium acetate, HPLC grade, was obtained from Fisher Scientific Co. (Fairlawn, NJ, USA). Glacial acetic acid, HPLC grade, was obtained from J.T. Baker (Phillipsburg, NJ, USA). Ultrapure grade tris(hydroxymethyl) aminomethane, Tris, was obtained from Aldrich (Milwaukee, WI, USA). HPLC grade solvents were used.

2.2. Instrumentation

The isocratic system consisted of the following components: a Kratos Spectroflow Model 400 pump and a Kratos Spectroflow Model 783 variable-wavelength detector (ABI Instruments, Ramsey, NJ, USA), and a Perkin-Elmer ISS-100 autosampler equipped with a 50-μl loop (Perkin-Elmer, Norwalk, CT, USA). Chromatograms recorded on a 10-mV strip chart recorder (Kipp and Zonen, Model BD-40, Delft, Netherlands) were visually monitored for performance of the chromatographic system. These were not used for quantitation. Chromatographic data were automatically acquired and processed using a Perkin-Elmer CLAS program.

2.3. Chromatographic conditions

Isocratic separation was achieved using an alkyl phenyl column (250 × 4.6 mm I.D., 5 μm packing) supplied by ES Industries (Marlton, NJ, USA). A precolumn, dry-packed with 37–53 μm silica gel (Whatman, Clifton, NJ, USA) was inserted in front of the injector to protect the analytical column. This is precautionary and was added at the start of the method development when the pH of the mobile phase was varied. The flow-rate of the mobile phase consisting of an aqueous buffer–acetonitrile–methanol (80:15:5, v/v) mixture was maintained at 0.8 ml/min. The aqueous buffer was 0.05 *M* ammonium acetate adjusted to pH 5.0 with glacial acetic acid. The analytes were detected at 295 nm using a setting of 0.010 AUFS.

2.4. Robotic system

A Zymark Py-robot (Zymark, Hopkinton, MA, USA) was used to automate the manual method. The robot is controlled by the Zymate System V controller which is linked to an AT and T 6286 WGS computer. The components and configuration of the robotic Py-setup are shown in Fig. 1. Two power and event controllers and two solvent delivery systems (Master Laboratory Stations, MLS) do not occupy Py-sections and are located peripheral to the controller.

2.5. Standard solutions

Note that BV-araU, BV-uracil and CV-araU are light-sensitive. Care must be taken to protect these compounds from light.

Stock solutions of BV-araU, BV-uracil, and CV-araU were prepared in methanol at concentrations of 500, 250 and 500 μg per ml,

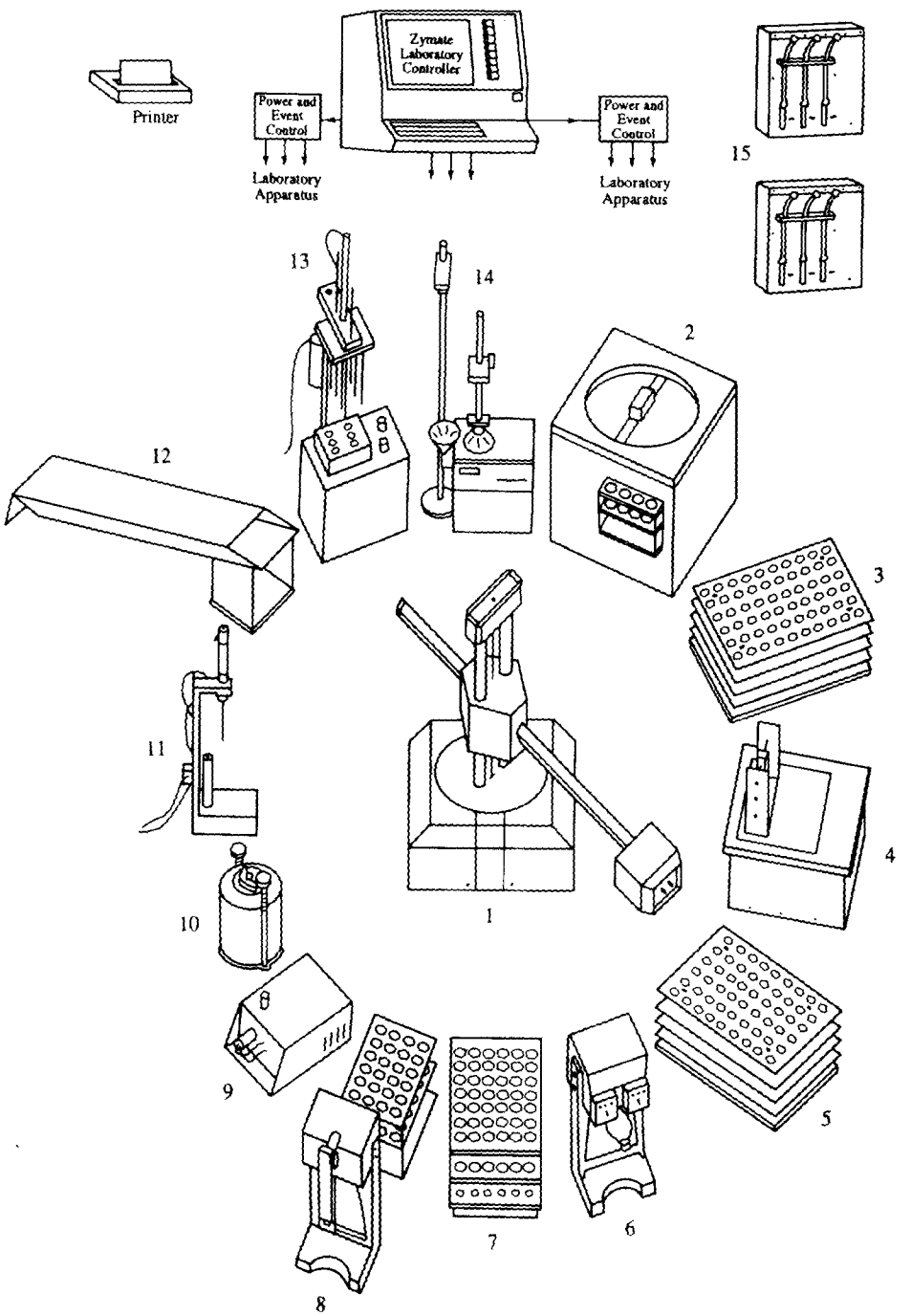

Fig. 1. Robotic set-up: (1) robot arm, (2) centrifuge, (3, 5) sample racks, (4) linear shaker, (6) general purpose hand, (7) aux. pipet tip rack, (8) pipetting hand, (9) LC injector, (10) capper, (11) liquid–liquid extractor, (12) disposal station, (13) evaporator, (14) custom dilute and dissolve, (15) master laboratory station.

respectively. These solutions were stable for at least ten weeks, provided they were refrigerated and protected from light. A secondary internal standard solution, prepared at 10 μg per ml in water, was used to spike the samples prior to extraction. An unextracted working standard solution was prepared daily to contain 1.0 μg of BV-araU, 0.40 μg of BV-uracil and 1.0 μg of CV-araU per ml of mobile phase. This solution was used to check the resolution of the chromatographic system.

2.6. Standard curve and control samples

Blank urine was spiked with 0.10–25 μg of BV-araU and 0.10–5.0 μg of BV-uracil per ml. These spiked samples were used to construct standard curves. In addition, control urine samples containing 10 μg of BV-araU and 4 μg of BV-uracil per ml were prepared. For every ten unknown samples extracted, one control sample was also extracted to monitor the assay.

2.7. Sample extraction

The described sample extraction can be performed either manually or automated using the Zymark robot. In either case the volume of buffer, the volume of organic solvent added and the volume of organic layer collected for evaporation are identical.

A 0.5-ml aliquot of the sample was transferred into a 15-ml polypropylene centrifuge tube (Becton–Dickenson Labware, Lincoln Park, NJ, USA) and 120 μl, containing 1.2 μg, of the internal standard was added to the sample. The sample was buffered with 1.0 ml of 0.05 *M* Tris buffer then extracted with 6.0 ml of ethyl acetate for 10 min using a linear reciprocating shaker. The sample was then centrifuged for ca. 10 min at 2800 *g* to separate the layers. Five ml of the ethyl acetate layer were transferred into a clean tube and evaporated to dryness at 50°C under a gentle stream of nitrogen. The residue was dissolved in 2.0 ml of mobile phase and 50 μl was injected onto the HPLC system.

2.8. Measurement and calculations

Chromatographic data management was automated using a P-E 3252 CLAS program.

The peak-height ratios of BV-araU and BV-uracil to internal standard were used to construct the standard curves. Unweighted least squares linear regression of the peak-height ratios as a function of the standard concentrations was applied to each standard curve. Concentrations of BV-araU and BV-uracil in the clinical samples were then determined from the respective regression equations.

3. Results and discussion

3.1. Method development

The chromatographic behaviors of the analytes were extensively studied and reported earlier. The chromatographic system described here is identical to that used for serum analysis [12]. The retention times of the internal standard, BV-araU, and BV-uracil are about 10, 13, and 17 min, respectively (Fig. 2A).

Both BV-araU and BV-uracil were extracted from human urine using a single extraction with ethyl acetate. By buffering the sample with Tris

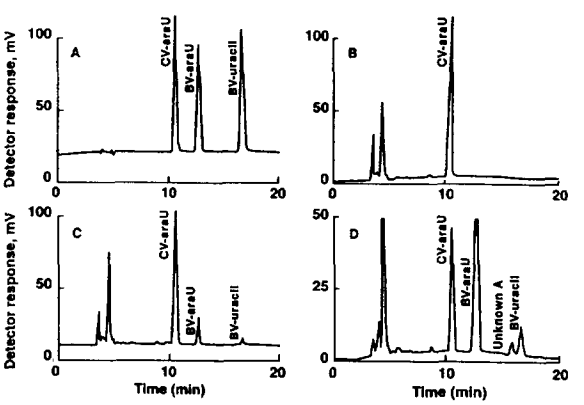

Fig. 2. Typical chromatograms: (A) working standard solution; (B) predose urine sample; (C) blank urine spiked with 0.5 μg of BV-araU and 0.10 μg of BV-uracil per ml; (D) urine sample containing 5.38 μg of BV-araU and 0.52 μg of BV-uracil per ml.

(pH 9.9), clean extracts devoid of interferences from endogenous substances were obtained (Fig. 2B). If the sample is not buffered, lots of interfering peaks were observed in the chromatograms. Quantitative recoveries were also obtained when 0.1 *M* potassium dibasic phosphate solution adjusted to pH 9.9 was used. However, the use of Tris buffer instead of potassium dibasic phosphate produced cleaner extracts. The cause of this difference was not investigated. The concentration of the Tris buffer is not critical. Anywhere from 0.007 to 0.10 *M* may be used.

The sample preparation was automated using a Zymark Py-robot equipped with all the necessary modules to carry out the assay. The robot also injected part of the reconstituted extract onto an HPLC system making the entire assay method fully automated.

3.2. Linearity

A standard curve in urine was prepared to contain 0.1–25 μg BV-araU and 0.1–5 μg BV-uracil per ml. The peak-height ratios of analyte to internal standard are directly proportional to the concentrations of analyte. Following the recommended procedure, typical linear regression results in the following equations:

$$\text{Conc of BV-araU } (\mu g/ml) = 5.2563\ (\text{Ratio}) - 0.06150,\ r^2 = > 0.9999$$

$$\text{Conc of BV-uracil } (\mu g/ml) = 4.6390\ (\text{Ratio}) - 0.01337,\ r^2 = > 0.9999$$

The standard-curve range may be extended to contain 50 μg of BV-araU and 10 μg of BV-uracil per ml without compromising the performance of the assay.

3.3. Selectivity

The sample extraction and chromatographic analysis were developed to produce a selective assay for the analytes. At least ten pre-dose samples from a clinical study were carefully evaluated for interferences in the assay. No interfering urine components were observed. A typical chromatogram of a drug-free urine sample is shown in Fig. 2B.

3.4. Sample recovery

The recoveries of BV-araU, BV-uracil, and CV-araU from urine were determined by comparing peak heights from spiked urine samples assayed accordingly versus peak heights of standard solutions prepared in mobile phase injected directly onto the analytical column. Absolute recoveries were 77 to 83% over the range of the standard curves. Taking into consideration the incomplete transfer of the organic layer, the recovery values suggest quantitative extraction of the analytes.

3.5. Lower and upper limits of quantitation

The lower limit of quantitation (LLQ) is defined as the lowest concentration in the standard curve that back-calculates with adequate precision and accuracy. Evaluation of the standard curves prepared over three days showed that the LLQ is 0.1 μg/ml for both BV-araU and BV-uracil. At this concentration, the accuracy is better than 90% and the C.V. is $< 10\%$.

During method validation, the standard curve was prepared to contain an upper limit of quantitation (ULQ) of 25 μg of BV-araU and 5 μg of BV-uracil per ml of urine. The standard concentrations were arbitrarily selected to cover the concentrations anticipated in clinical samples. In limited trials, the ULQ has been extended to 50 μg of BV-araU and 10 μg of BV-uracil per ml of urine without compromising the performance of the assay. Samples that contain analyte concentrations higher than the ULQ can be assayed after appropriate dilution with water.

3.6. Accuracy and precision

The accuracy and precision of the assay were determined by spiking both parent drug and metabolite into drug-free urine at various concentrations. Recovery, precision, and accuracy

were constant over the whole concentration range studied. For BV-araU in the range 0.1–25 μg/ml, the overall mean recovery was 100.0 ± 1.9% and for BV-uracil over the concentration range 0.1–5 μg/ml, the overall mean recovery was 100.5 ± 1.4%. No bias was observed over the range of concentrations studied.

The intra- and inter-day assay variations were assessed with six spiked urine samples prepared with varying amounts of BV-araU and BV-uracil assayed four times each on three different days ($n = 12$). Statistical evaluation of the results established good accuracy and precision of the method. For the three-day analyses, the mean daily recovery ranged from 98.8 to 103% for each analyte. On each day, the coefficient of variation (C.V.) was typically less than 5% at concentrations other than the LLQ. At the LLQ, the C.V.s for BV-araU and BV-uracil were 7 and 9%, respectively. The variance components were estimated using a two-way analysis of variance model with input as a fixed factor and day as random factor. In general, no inter-day variation was observed for the determination of either analyte.

During a typical week ($n = 5$), the slopes of the standard curves for both BV-araU and BV-uracil were quite reproducible with a C.V. of $< 3\%$ for each analyte further supporting the precision of the assay method.

3.7. Stability

The robotic assay is usually run unattended overnight. In order to assess the stability of the analytes in urine, spiked samples were deliberately left on the robotic rack overnight at ambient conditions prior to analysis. The results showed that the analytes are stable at ambient conditions for at least 24 h.

The stability of both analytes in frozen human urine was determined by periodic analysis over a span of sixteen weeks. The results indicated that no significant degradation occurred over the sixteen-week period. In addition, select clinical samples were assayed within one week of sample collection and then re-assayed after four weeks. The results further indicate that the analytes are stable in frozen urine. Furthermore, the analytes are also stable through at least three freeze-thaw cycles.

3.8. Effect of sample dilution prior to extraction

The effect of sample dilution was investigated with spiked samples as well as actual clinical samples. Samples were diluted with water at ratios of 1:2, 1:5, and 1:10 prior to extraction. The results indicate that the extent of dilution does not affect the assay results. Using a one-to-ten dilution, as high as 500 μg of BV-araU and 100 μg of BV-uracil per ml of urine can be measured.

3.9. Automation of the method

A Zymark Py-robot was used to automate the sample extraction. The hardware connections of the robot are depicted in Fig. 1. The robot was programmed to inject part of the reconstituted extract onto an HPLC system which was located peripheral to the core system. Since data acquisition was also automated, the entire method was fully automated.

The extraction procedure described in section 2.7 was optimized manually then automated such that the robot mimicked the manual procedure.

In order to compare the performance characteristics of the manual method versus using the robot, urine samples spiked at six different levels of both BV-araU and BV-uracil were assayed three times manually and four times with the robot. By comparing the accuracy and precision obtained, both manual and robotic methods were shown to be equivalent. In general, mean recoveries ranged from 96 to 104% except at the LLQ for BV-uracil where the mean recovery using the robot was 110%. The C.V.s for both methods were $\leq 6\%$ at all levels. This was adequate for pharmacokinetic studies. Because of the huge number of clinical samples that are submitted for analysis, the robot is routinely used in our laboratories.

Table 1
Typical assay results following BID dose of 50 mg BV-araU

Collection period (h)	BV-araU (μg/ml)	BV-uracil (μg/ml)
Subject A		
0–3	27.0	0.50
3–6	35.2	0.41
6–12	44.4	0.59
12–24	11.7	0.80
24–48	2.3	0.36
Subject B		
0–3	53.6	0.55
3–6	88.8	0.85
6–12	40.6	0.97
12–24	10.5	0.88
24–48	1.7	0.33

BID = twice a day.

3.10. Application

The method has been successfully used to assay human urine samples from pharmacokinetic and clinical samples. The chromatogram of a urine sample, Fig. 2D, shows one other peak (unknown A) with a retention time just before the BV-uracil peak. This could be another metabolite with a concentration less than the BV-uracil level. Typical concentration–time profiles for samples from a Phase I study are shown in Table 1.

4. Conclusions

The method described here is selective and suitable for the analysis of clinical samples. Both manual and robotic sample extractions gave comparable results. If a robot is not available, the method can be semi-automated by preparing the extracts manually and performing the chromatographic analysis automatically. For practical purposes, enough extracts are prepared in one day to run the chromatographic analysis overnight. Alternatively, the extracted residues may be frozen overnight and the residues reconstituted in mobile phase and chromatographed the following day. The frozen residues are stable for at least 48 h. The automated assay with the robot is very convenient when a huge number of samples has to be assayed. The use of the robot saves the analyst from performing tiring and monotonous work.

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