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Short communication

Determination of etoposide phosphate intermediates by gradient liquid chromatography using postcolumn derivatization with cuprammonium hydroxide

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Abstract

Allyl 4,6-*O*-ethylidene-β-D-glucopyranoside is an intermediate used in the synthesis of etoposide phosphate, a water-soluble derivative of the antineoplastic drug etoposide. In this paper, a method for the determination of allyl 4,6-*O*-ethylidene-β-D-glucopyranoside and related glucopyranosides is described. Samples are analyzed by gradient high-performance liquid chromatography using postcolumn derivatization with cuprammonium hydroxide. The method is used to monitor the ethylidenation reaction of allyl β-D-glucopyranoside with acetal to form allyl 4,6-*O*-ethylidene-β-D-glucopyranoside. Samples are chromatographed on an octadecyl-bonded phase column with aqueous acetonitrile. Triethylamine is added to the mobile phase to accelerate mutarotation and suppress anomeric separations. The column effluent is mixed via a postcolumn tee with cuprammonium hydroxide and monitored with ultraviolet detection at 310 nm.

Keywords: Pharmaceutical analysis; Derivatization, LC; Etoposide phosphate; Glucopyranosides; Carbohydrates; Cuprammonium hydroxide

1. Introduction

Etoposide phosphate (EP) is a water-soluble prodrug for etoposide (VP-16), an anticancer drug derived from the natural product podophyllotoxin. EP is used in the treatment of small-cell lung cancer, leukemia, and testicular cancer. In animals or in vitro, EP is rapidly and completely converted to VP-16 [1]. The mechanism of action for VP-16 suggests that cytotoxicity results from extensive DNA damage including single- and double-strand breaks [2]. Unlike VP-16, the water solubility of EP allows it to be formulated without polysorbate 80, ethanol, or polyethylene glycol. As a result, EP may be prepared at higher concentrations and administered over a shorter period of time than VP-16.

Allyl 4,6-*O*-ethylidene-β-D-glucopyranoside (AEG) is an intermediate in the synthesis of EP. It is formed by the reaction of allyl β-D-glucopyranoside (AG) in acetonitrile and toluene with acetal in the presence of acid catalyst [3]. Four potential process impurities [β-D-glucose, 4,6-*O*-ethylidene-β-D-glucose (EG), methyl 4,6-*O*-ethylidene-β-D-glucopyranoside (MEG), and ethyl 4,6-*O*-ethylidene-β-D-glucopyranoside

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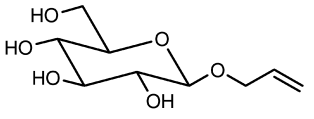
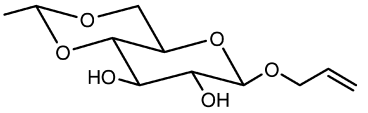
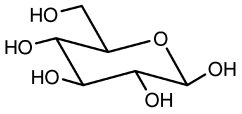


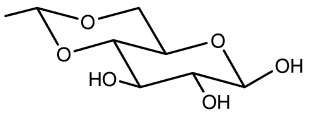
allyl β-D-glucopyranoside
(AG)



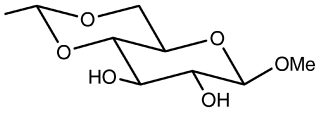
allyl 4,6-*O*-ethylidene-β-D-glucopyranoside
(AEG)



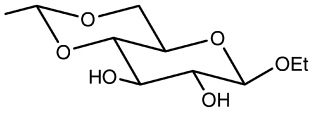
β-D-glucose



4,6-*O*-ethylidene-β-D-glucose
(EG)



methyl 4,6-*O*-ethylidene-β-D-glucopyranoside
(MEG)



ethyl 4,6-*O*-ethylidene-β-D-glucopyranoside
(EEG)

Fig. 1. Chemical structures.

(EEG)] exist. Chemical structures are shown in Fig. 1.

During development of the synthesis, the reaction was monitored by thin-layer chromatography (TLC). Nuclear magnetic resonance (NMR) was used to evaluate product quality. Prior to the running of pilot plant scale large batch reactions, it was necessary to develop a more accurate method for quantitative determination of reaction progress, product purity, and yield loss to the filtrate.

Excellent resolution for the compounds of interest was afforded by chromatography on an octadecyl bonded silica column using a gradient from 4 to 20% acetonitrile in aqueous solution. The addition of triethylamine (TEA) to the mobile phase was required in order to prevent anomeric separations [4] of the sugars which were not protected at the C-1 position, e.g., glucose and EG.

The samples were derivatized postcolumn with cuprammonium hydroxide using the conditions described by Grimble et al. [5]. This system provided good sensitivity and was not adversely effected by the gradient profile.

The method described is rugged and reproducible. It was used to assay hundreds of samples including reaction streams from a 10-week pilot plant campaign requiring continuous analytical support.

2. Experimental

2.1. Chemicals

High-purity water was obtained from Burdick and Jackson (Muskegon, MI, USA). HPLC-grade acetonitrile, certified ACS cupric sulfate, and certified ACS PLUS ammonium hydroxide (29.9%, w/w, as ammonia) were purchased from Fisher (Fair Lawn, NJ, USA). Triethylamine, 99% was obtained from Aldrich (Milwaukee, WI, USA). Glucose monohy-

drate was purchased from A.E. Staley Manufacturing (Decatur, IL, USA). All other sugars were synthesized in the laboratory.

2.2. Instrumentation and conditions

The high-performance liquid chromatography (HPLC) system consisted of a Hewlett-Packard 1050 series quaternary pump, an on-line degasser, and an autosampler (Palo Alto, CA, USA) with a Waters 486 detector (Milford, MA, USA). The Hewlett-Packard components were controlled by a Hewlett-Packard Vectra 486/66XM personal computer loaded with Hewlett-Packard ChemStation software. The detector signal was routed through a Hewlett-Packard 35900C multichannel interface. A Waters 510 was used as the postcolumn pump with a high-sensitivity noise filter accessory (Waters P/N WAT025200) installed between the pump and the postcolumn tee (Waters P/N WAT075215). A 10 cm piece of 0.01 in. I.D. stainless steel tubing was installed between the postcolumn tee and the detector (1 in.=2.54 cm). Chromatography was performed using a GL Sciences Inertsil ODS-2 column (25 cm×4.6 mm I.D.) purchased from Jones Chromatography (Lakewood, CO, USA).

Mobile phase A consisted of acetonitrile–water–triethylamine (4:96:0.05, v/v). Mobile phase B consisted of acetonitrile–water–triethylamine (20:80:0.05, v/v). The analytical flow-rate was 1 ml/min and the gradient profile was as follows: from 0 to 5 min, composition was maintained at 100% A. At 5 min, a 2 min linear gradient to 100% B was initiated. From 7 to 16 min, 100% B was maintained. At 16 min, a reverse step gradient to 100% A was executed. 10 min were then allowed for column equilibration at 100% A.

The postcolumn reagent stock solution was prepared by dissolving 25 g of cupric sulfate in 500 ml of water and adding 500 ml of ammonium hydroxide. The working solution was prepared by combining 100 ml of the stock solution with 250 ml of ammonium hydroxide and 650 ml of water. The postcolumn reagent flow-rate was 0.5 ml/min.

The autosampler was programmed to deliver 100 μl injections. Samples were detected at 310 nm.

2.3. Sample preparation

The progress of the ethylidenation reaction was monitored at 1 h intervals by evaporating 0.3 ml of reaction mixture to dryness under a stream of nitrogen. The residue was then dissolved in 10 ml of water. The reaction was deemed complete when less than 3% of the starting material was detected.

The loss of product to the filtrate was determined by evaporating 0.5 ml of sample to dryness under a stream of nitrogen. The residue was then dissolved in 10 ml of water.

The quality of isolated product was ascertained by accurately preparing sample solutions at 2 mg/ml in acetonitrile–water (4:96, v/v). Samples were quantitated versus a standard of known purity.

3. Results and discussion

It is important to note that if the time for equilibration at initial conditions is extended beyond the 10 min specified in the method, retention times for the subsequent injection are not consistent. Increasing the equilibration time results in decreased retention times. It was also observed that decreasing the concentration of TEA in the mobile phases results in increased retention times. This may result from a buildup of triethylamine on the column due to the comparatively low solubility of TEA in water versus in acetonitrile. It is possible that during each gradient, as the acetonitrile content is increased to 20%, excess TEA is washed from the column.

Several pre- and postcolumn derivatizations found in the literature, such as reaction with tetrazolium blue [6] or 2-cyanoacetamide [7], were evaluated. In all cases, however, the desired reaction did not proceed for the C-1 protected sugars. Additionally, the sugars in question were not amenable to electrochemical detection.

The postcolumn reaction developed by Grimble et al. [5] is based on the complexation of 1,2-diols with cuprammonium to give a UV absorptive product detectable between 285 and 310 nm. As predicted by structure, all of the compounds of interest were amenable to this detection scheme.

In Grimble et al.'s paper, the postcolumn reagent

was delivered through the use of a gas pump and nitrogen pressure. For the sake of convenience, a dual piston HPLC pump was used in this laboratory. In order to reduce baseline noise resulting from pump pressure pulsation, an external pulse dampener was installed between the postcolumn pump and the postcolumn tee. Noise from the HP 1050 analytical pump was minimized by optimizing the pump's user selectable compressibility factor to give a pressure ripple of 0.1%.

The method conditions were further refined through optimization of the postcolumn flow-rate and detection wavelength. Postcolumn flow-rates were examined from 0.5 to 1.2 ml/min. Greater sensitivity

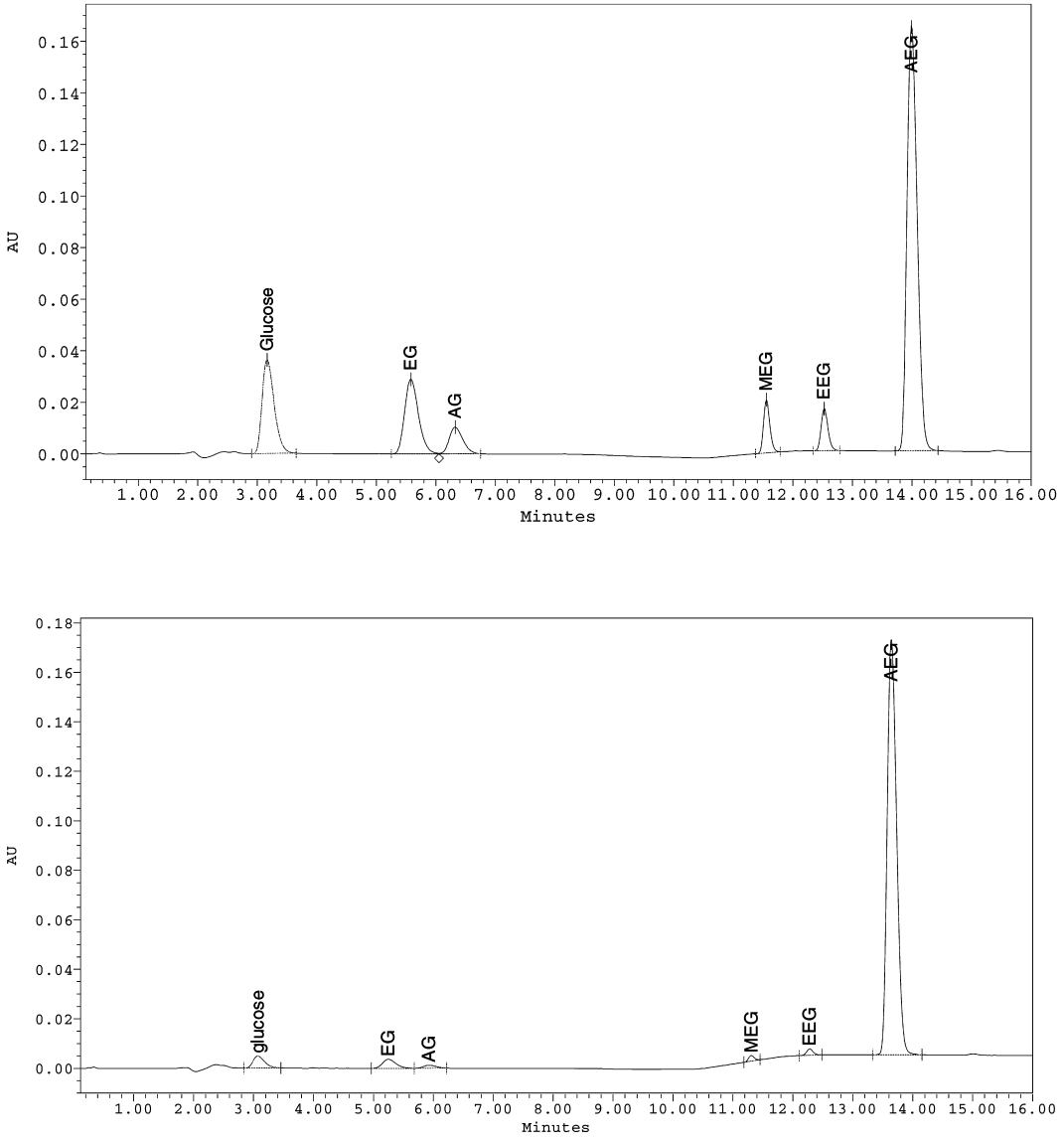


Fig. 2. Chromatograms of AEG at 2 mg/ml spiked with several known impurities at 0.1 mg/ml and a typical ethylidenation reaction mixture sample. Inertsil ODS-2, 250×4.6 mm, 1 ml/min CH_3CN–water–TEA (4:96:0.05) for 5 min followed by a 2 min gradient to CH_3CN–water–TEA (20:80:0.05), 100 μl injection volume. UV detection at 310 nm after postcolumn derivatization with cuprammonium hydroxide at 0.5 ml/min.

was obtained at lower flow-rates due to less dilution of the sample in the detector cell. At 0.5 ml/min, an excess of postcolumn reagent to ensure complete complexation was still delivered to the postcolumn tee.

Detection wavelengths ranging from 285 to 310 nm were examined. While sensitivity increased as wavelength decreased, a concomitant increase in background noise was also observed. Therefore, 310 nm was selected as the detection wavelength. Typical chromatography for a standard mixture and a reaction mixture is shown in Fig. 2.

Linearity of detector response for AEG was evaluated by plotting peak area vs. sample concentration over the range of 1.0 to 2.5 mg/ml. The correlation coefficient for the linear regression equation was 0.9992.

The linearity of detector response for glucose, AG, EG, MEG, and EEG was determined over the range of 2.0 to 100 μg/ml. Correlation coefficients for each of the liner regression equations were 0.9999.

The quantitation limit for AEG and each of the potential impurities was estimated by multiplying the signal-to-noise ratio observed in blank injections by a factor of 10. This resulted in a quantitation limit of 2 μg/ml for each of the sugars. Detection limits were estimated in similar fashion by applying a signal-to-noise ratio of 3:1. This resulted in a detection limit of 0.6 μg/ml for each of the sugars. These limits were verified by injection of sample solutions prepared at the estimated limits.

The injection precision for AEG was determined by making six injections of a 2.0 mg/ml standard solution. The relative standard deviation (RSD) for the six injections was 0.4%. Injection precision for each of the potential impurities was determined by making six injections of a 100 μg/ml standard solution. The RSDs for the six injections ranged from 0.3 to 4.1%.

4. Conclusion

In conclusion, a HPLC method for the determination of etoposide phosphate intermediates using postcolumn derivatization with cuprammonium hydroxide was developed. The conditions described are applicable to the analysis of AEG and related glucopyranosides, including those protected at the C-1 position. The combination of gradient reversed-phase liquid chromatography and postcolumn derivatization with cuprammonium hydroxide is a useful technique for the determination of glucopyranosides that cannot be analyzed using conventional carbohydrate methodologies.

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