Journal of Chromatography, 276 (1983) 349—357
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1733

AUTOMATED LIQUID CHROMATOGRAPHIC ANALYSIS OF THE ANTI-TUMORIGENIC DRUGS ETOPOSIDE (VP 16-213) AND TENIPOSIDE (VM 26)

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(First received January 17th, 1983; revised manuscript received March 23rd, 1983)

SUMMARY

A method is described for the fully automated analysis of large numbers of 1—2 ml serum and plasma or urine samples containing the anti-tumorigenic drugs etoposide and teniposide and their aglycone. The blood samples are hydrolysed by a proteolytic enzyme, subtilisin A, prior to preconcentration on a small precolumn. The hydrolysis step serves both to release the strongly protein-bound drugs and to prevent clogging of the chromatographic system. On-line preconcentration is carried out with precolumns packed with PRP_1, a microparticulate divinylbenzene—styrene copolymeric sorbent. Chromatography takes place, after column switching, in a C_{18}/methanol—water system. After a post-column clean-up step using continuous extraction with dichloroethane in an autoanalyzer system, native fluorescence of these analytes is used for detection of the drugs. Recovery of etoposide and teniposide from spiked serum and plasma samples was 100%.

Calibration curves of etoposide and teniposide typically show correlation coefficients of 0.9994 over a two-to-three order linear range. The detection limit of etoposide is approx. 8 ng per sample. Repeatability was found to be excellent. Unattended overnight routine analysis is possible without any problems. This method, considering optimal sample throughput, reliability and selectivity, competes favourably with existing techniques for the analysis of etoposide and teniposide.

INTRODUCTION

Recently several liquid chromatographic procedures for the determination of the antineoplastic agents etoposide (VP 16-213) [1] and teniposide (VM 26) have been described [2—5]. In these procedures off-line preconcentration by

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liquid—liquid extraction and evaporation to dryness is carried out, followed by reversed-phase chromatography in a C_{18}/methanol—water system and detection either by UV absorption at 254 and 280 nm or by native fluorescence. Recently, electrochemical detection has also been described [6].

A major drawback of the procedures reported in the literature is the time-consuming and cumbersome off-line sample preparation. Further, special care has to be taken to ensure that quantitative extraction of the strongly protein-bound drugs [1] takes place and that the structure of the drugs remains unimpaired.

In this paper we describe the use of mild protein hydrolysis by a proteolytic enzyme as a sample preparation step to release the drugs and to allow repeated injection of large (0.2—2 ml) amounts of blood samples directly into the chromatographic system (cf. ref. 7). The hydrolysed serum and plasma samples are preconcentrated on a short precolumn and, after column switching, analysed in a C_{18}/methanol—water system followed by continuous post-column liquid—liquid extraction as a clean-up step, and fluorescence detection.

EXPERIMENTAL

Liquid chromatography

Automated preconcentration and high-performance liquid chromatographic (HPLC) analysis were carried out with a Kontron (Zürich, Switzerland) liquid chromatograph, consisting of two Model 410 pumps, an MSI 660 autosampler, a Model 200 programmer, a prototype of the MCS 670 column switching apparatus and a Perkin-Elmer (Norwalk, CT, U.S.A.) 3000 fluorescence detector set at λ_{ex} = 230 nm and λ_{em} = 328 nm. Signals were recorded on a W + W 900 (Kontron) recorder. Quantitation of the signals was carried out manually. The apparatus used is schematically shown in Figs. 1 and 2.

The post-column extraction system consisted of a Technicon (Tarrytown, NY, U.S.A.) AutoAnalyzer pump Model II, equipped with Acidflex tubing in order to pump the extraction solvent dichloroethane. The flow-rate of the reagent stream was 0.6 ml/min, the flow-rate through the fluorescence detector

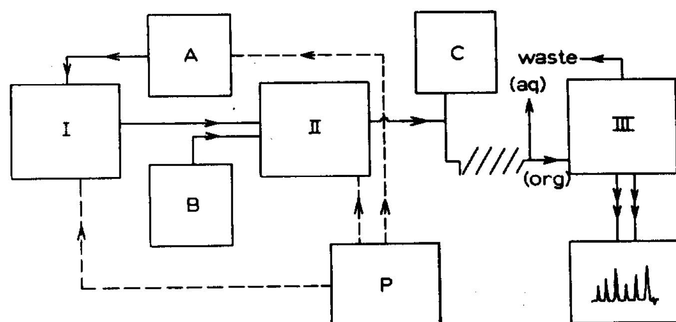

Fig. 1. Scheme of the apparatus for automated analysis of etoposide and teniposide. I, Autosampler; II, column switching apparatus; III, fluorescence detector; A and B, HPLC pumps; C, autoanalyzer pump; P, programmer.

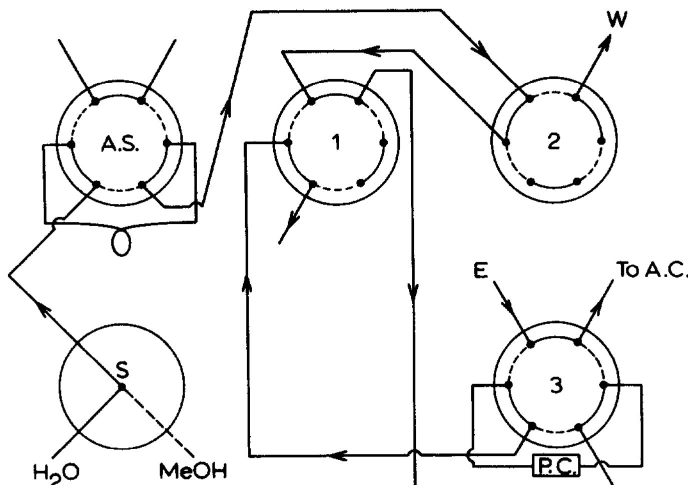

Fig. 2. Switching valve configuration. A.S., autosampler injector equipped with 1.6-ml loop. S, low-pressure selector valve. 1, 2, 3, high-pressure switching valves. A.C., analytical column. E, eluent, P, precolumn. W, waste. The indicated configuration (solid line) applies for the preconcentration step.

0.3 ml/min. Extraction took place in 2 mm I.D. standard Technicon glass reactors with ten or twenty coils. The phase separator was a modified Technicon one with PTFE insert [8].

Chromatography was carried out with a 125 × 4.0 mm I.D. LiChroCart column, prepacked with 10-μm LiChrosorb RP-18 (Merck, Darmstadt, G.F.R.) or with a 100 × 4.1 mm I.D. stainless-steel column, home packed with 5-μm Nucleosil C_{18} (Macherey & Nagel, Düren, G.F.R.). Methanol—water mixtures with or without 1% (v/v) glacial acetic acid were used as mobile phase. The mobile phase flow-rate was 1 ml/min.

Preconcentration was carried out on home-made 2 × 4.6 mm I.D. or 10 × 2 mm I.D. precolumns which were slurry-packed by micro-spatula or by syringe [9] with Nucleosil C_{18} or PRP_1 (Hamilton, Bonaduz, Switzerland), a microparticulate (10-μm) divinylbenzene—styrene copolymer.

Chemicals

Etoposide, teniposide and aglycone were received as a gift from J.M.S. van Maanen (Dutch Cancer Institute, Amsterdam, The Netherlands). The structures of these compounds are given in Fig. 3. Aglycone was used as internal standard for etoposide.

Subtilisin A was purchased from Novo Industry A/S (Bagsvaerd, Denmark), and proteinase K from Merck. All solvents were purchased from Baker (Deventer, The Netherlands) and were of analytical grade. All aqueous solutions were prepared with demineralized water, treated in a Milli Q (Millipore, Bedford, MD, U.S.A.) ultrafiltration system. Eluents and urine samples were filtered through an all-glass filtration apparatus with 0.2-μm filters (Millipore) and degassed under vacuum before use. Frozen sterilized calf serum was

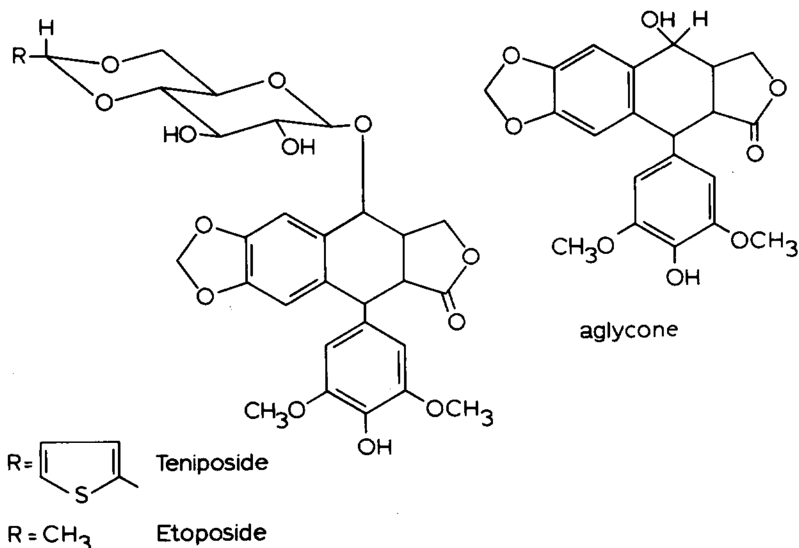

Fig. 3. Structures of etoposide, teniposide and aglycone.

obtained from Flow Laboratories (Irvine, Great Britain). Frozen human plasma was received as a gift from the blood transfusion service of the Onze Lieve Vrouwe Gasthuis (Amsterdam, The Netherlands).

PROCEDURE

Enzymatic hydrolysis

Aliquots of water, plasma or serum samples spiked with etoposide and/or teniposide were incubated with equal amounts of 1 mg/ml aqueous subtilisin A solution for 15 min at 50°C. Incubation with proteinase K took place for 10 min at ambient temperature.

Column switching

Automated preconcentration, precolumn clean-up and precolumn switching were carried out according to the procedure described in Table I. The individual steps of the procedure were designed to effect optimal performance regarding analysis time, sample throughput, recovery of analyte, sample clean-up, absence of cross-contamination and absence of clogging problems.

Hydrolysed blood and/or filtered urine samples were put in the tray of the autosampler and subsequently transferred to the precolumn using water as the carrier stream at a flow-rate of 1 ml/min (see Fig. 1). The precolumn was then backflushed with 4 ml of water at a flow-rate of 2 ml/min to remove large protein fragments, or other, hydrophilic, matrix compounds and to effect a partial sample clean-up. The precolumn was then switched on-line with the analytical column and backflushed for 30 sec using a methanol—water mixture. Sample analysis took 5—10 min. Meanwhile, the precolumn was backflushed with 6 ml of methanol and a flow-rate of 2 ml/min to remove strongly retained

TABLE I

PROGRAM FOR THE AUTOMATED ANALYSIS OF ETOPOSIDE, TENIPOSIDE AND AGLYCONE USING A PRECOLUMN

Time (min)	Event	
0	start FILE 12	
0	start FILE 11	
0	start autosampler	Filling sample loop
2.7	start FILE 10 (% B 100, flow 1 ml/min, AUX 2 DUR 4)	Preconcentration on PC* } with water
4.7	AUX 1 DUR 7.5 (flow 2 ml/min)	Backflush washstep } with water
6.7	AUX 3 DUR 0.5	Elution of sample from PC
	AUX 5 DUR 0.01	Changing from water to methanol (to waste)
	flow 10 ml/min	
7.2	AUX 2 DUR 3 flow 2 ml/min	Backflush washstep of PC with methanol**
10.2	AUX 5 DUR 0.01	Changing from methanol to water (to waste)
	flow 10 ml/min	
10.7	AUX 2 DUR 1 flow 2 ml/min	Backflush PC with water to remove methanol
11.7	flow 0	
11.8	END	End/start again at t_0 depending on number of samples programmed in file 12

*PC = precolumn.
**Extra wash step to remove plasma components from the precolumn that would interfere with a subsequent etoposide analysis at high sensitivity.

compounds and then reconditioned with 2 ml of water at a flow-rate of 1 ml/min. The total program for the Model 200 microprocessor is given in Table I. The switching valves configuration is depicted in Fig. 2.

RESULTS AND DISCUSSION

Selection of packing materials

Preconcentration of etoposide from aqueous solution on a short (2 × 4.6 mm I.D.) precolumn packed with C_{18} chemically bonded silica could not be carried out with 100% recovery as breakthrough occurred even after 2 ml, due to insufficient retention. Since it is necessary to backflush the precolumn with a few milliliters of water after sampling 1—2 ml of serum or plasma (cf. ref. 7), a breakthrough volume of 2 ml is not sufficient for the analysis of patient samples. Therefore, PRP_1 was used instead of the C_{18} material as this sorbent displays 10—20 times higher retention towards aromatic compounds than C_{18} packing materials [10]. With short (2 × 4.6 mm I.D.) PRP_1 precolumns, breakthrough of etoposide was not observed even after flushing with 20 ml of water. As chromatography of etoposide was not very efficient on PRP_1, C_{18} analytical columns were used in combination with PRP_1 precolumns.

Enzymatic hydrolysis

The necessity of enzymatic hydrolysis prior to preconcentration of blood samples in order to prevent clogging of the present chromatographic system was demonstrated as follows. Already after one injection of 0.5 ml of preconcentrated plasma (1:1 diluted with water) the pressure drop over the analytical column increased by 6 MPa. With subtilisin A-treated samples no increase of pressure was observed even after several hours of continous operation and passage of 30-ml hydrolysed blood samples. Upon injection of 0.5 ml of non-hydrolysed (1:1 diluted) plasma the recovery of etoposide was only 50%, while 100% recovery was obtained after protein hydrolysis with subtilisin A or proteinase K. Incubation of etoposide with subtilisin A at 55°C for 2 h was found not to affect the structure of the drug. The advantage of proteinase K over subtilisin A is that it can be used at ambient temperature. A disadvantage is its high price. For the present application, subtilisin A and proteinase K were found to be equally effective in protein hydrolysis. Further experiments were all carried out with subtilisin A.

Detection

Unfortunately, clean-up of the preconcentrated samples via washing with 6 ml of water was not sufficiently effective to remove all the compounds which interfere with the detection of the drugs by means of UV or fluorescence monitoring. Therefore a post-column extraction step with dichloroethane was introduced which was found to provide extremely low fluorescence background signals with hydrolysed blood samples and reasonably low background signals with urine samples. Due to the higher background with urine samples the detection limit was somewhat higher (30 ng) than with hydrolysed (1:1 diluted) blood samples (8 ng) of the same volume (1.6 ml). The concentration range of etoposide in human plasma during the first 24 h after an intravenous therapeutic dose of 100 mg/m^2 typically is 20—0.5 μg/ml [3, 5].

Quantitation

Internal standardization. Both teniposide and aglycone can be used as internal standards in the analysis of etoposide. Fig. 4 shows chromatograms obtained after duplicate injection of plasma samples spiked with etoposide and teniposide. Separation of aglycone and etoposide requires a lower methanol content in the eluent (45%) than that of teniposide and etoposide (55% methanol). Since the use of less methanol causes a lower noise level in the extraction detector, the use of aglycone as internal standard for etoposide is to be preferred. Calibration curves for subtilisin A-treated plasma and serum samples spiked with etoposide using either teniposide or aglycone as internal standard were found to be linear over the concentration range 0.01—25 μg/ml. The correlation coefficient typically was 0.9994 (n = 10). The detection limit of etoposide, in plasma, is approx. 8 ng (see Fig. 5). This figure is of the same order of magnitude as with HPLC and electrochemical detection [6] and better than with HPLC and fluorescence detection using liquid—liquid extraction as sample pretreatment step (150 ng/ml) [3].

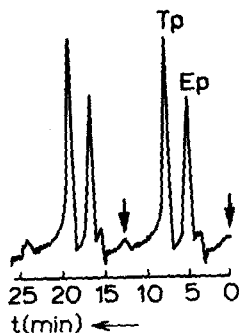

Fig. 4. Duplicate injection (see Table I) of 1.6 ml of a mixture of etoposide (Ep) and teniposide (Tp). Amounts injected: teniposide, 1.09 μg; etoposide, 503 ng. Chromatographic conditions: column, LiChroCart C_{18}; fluorescence detection after post-column extraction; mobile phase, methanol—water (55:45, v/v); flow-rate, 1 ml/min. Further conditions, see text.

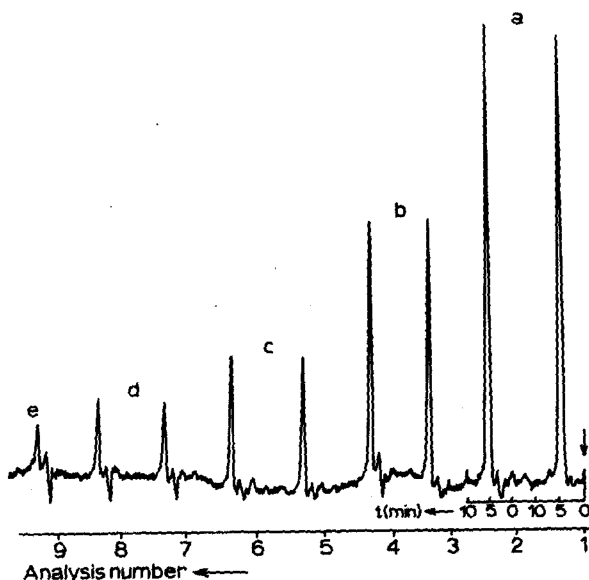

Fig. 5. Automated determination of the calibration curve and detection limit of etoposide in 0.5 ml of hydrolysed plasma samples. Amount of etoposide (in ng): (a) 257, (b) 158, (c) 82, (d) 43, (e) 23. Procedure: see text. Chromatographic conditions: column, 5-μm Nucleosil C_{18}; mobile phase, methanol—water—glacial acetic acid (46:64:1), pH 3.3; flow-rate, 1 ml/min. Further conditions, see Fig. 4.

External standardization. Plotting of peak area versus concentration of etoposide in hydrolysed plasma samples and aqueous solutions yielded identical calibration curves. In other words, the recovery of the drug from the hydrolysed blood samples is quantitative. This excellent result is caused by the efficient action of subtilisin A.

Plotting of peak heights instead of areas resulted in calibration curves with steeper slopes for the aqueous samples than for the blood samples (see Fig. 6). This is caused by a 85% increase in peak width upon preconcentration from

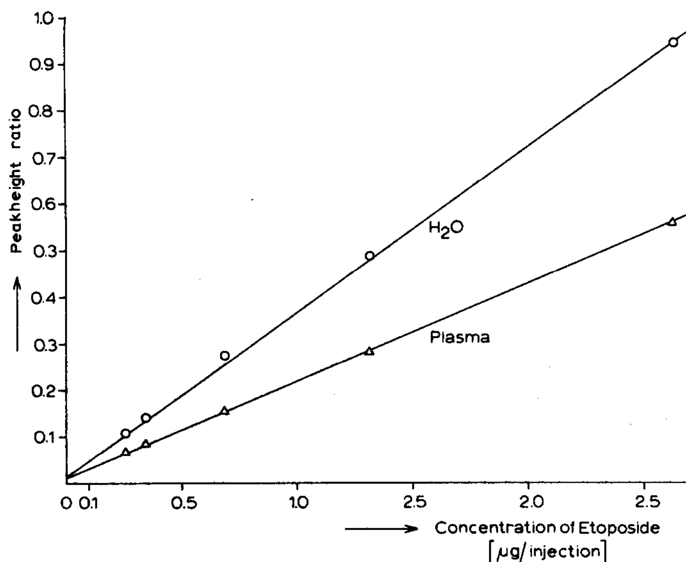

Fig. 6. Calibration curves of etoposide in water (○) and hydrolysed plasma (△). Ordinate: ratio of peak height of etoposide/aglycone. Abscissa: concentration of etoposide (µg/ml). Sample size: 1.6 ml.

plasma as compared to preconcentration from purely aqueous samples. The additional peak broadening is due to the fact that the surface of the sorbent in the precolumn is largely occupied by matrix constituents, which causes spreading of the analyte zone. With aged repeatedly frozen and thawed serum samples, which are known to contain smaller protein fragments [7], additional band broadening was much less than with fresh plasma samples.

Routine analysis

The present method has been used routinely without problems for several months. The repeatability was ± 2.2% (mean relative S.D.) (n = 10). Due to fluctuations in the response of the fluorescence detector, daily calibration was necessary. Using the program given in Table I, unattended operation was possible even with relatively large injection volumes, without increase of pressure or baseline shifts. As the precolumns are easily replaced, newly packed precolumns were installed every day, in order to prevent problems due to loss of performance of the stationary phase material. In all probability, however, precolumn lifetime is much longer than a single day.

One of the interesting aspects of the use of (inexpensive) precolumns is the possibility of sample collection and storage. Etoposide-spiked, hydrolysed plasma samples were preconcentrated and the precolumns stored for 40 h at −20°C. After analysis, the recovery was still found to be 100%.

CONCLUSIONS

A selective and sensitive method for the automated liquid chromatographic analysis of total etoposide and teniposide in biological samples has been devel-

oped. The method can be carried out without cumbersome sample preparation steps even with repeated injection of relatively large (1—2 ml) serum and plasma samples. Preconcentration is carried out on short (2 × 4.6 mm I.D.) PRP_1 precolumns and is followed by a backflush wash step with water and subsequent separation in a C_{18}/methanol—water system. Selective detection takes place after post-column extraction to 1,2-dichloroethane using the native fluorescence of the drugs. Continuous, unattended routine analysis of enzymatically hydrolysed blood samples is possible. The detection limits are comparable to those described in the literature, but the selectivity of the present method is much better.

The principle of on-line sample clean-up by a post-column extraction system presented here may well be more widely applicable and should be further investigated.

ACKNOWLEDGEMENTS

J.M.S. van Maanen (Dutch Cancer Institute, Amsterdam, The Netherlands) is thanked for the gift of etoposide, teniposide and aglycone. Kontron Ltd. (Zürich, Switzerland) is thanked for the loan of the column switching unit and other apparatus to carry out this work.

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