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AUTOMATED REVERSED-PHASE CHROMATOGRAPHIC ANALYSIS OF ETOPOSIDE AND TENIPOSIDE IN PLASMA BY USING ON-LINE SURFACTANT-MEDIATED SAMPLE CLEAN-UP AND COLUMN-SWITCHING

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SUMMARY

An automated high-performance liquid chromatographic method for the plasma assay of two neutral drugs, etoposide and teniposide, involving direct plasma injection is presented The problematic nature of protein precipitation has been circumvented by adding the anionic surfactant sodium dodecyl sulphate to the plasma at a final concentration of 38 m*M* Plasma samples are loaded on to a clean-up column with an aqueous mobile phase with which the analyte(s) is (are) retained, whereas the solubilized plasma proteins are flushed to waste Next, the retained compounds are eluted from the clean-up column on to the analytical column by using the chromatographic mobile phase with a higher elution capacity The column-switching technique is used to achieve an automated assay At least 10 ml of plasma, representing 100 repeated injections of 100 μl or five repeated injections of 2 ml, can pass through the clean-up column without increasing the back-pressure The recovery increased considerably from 10–30% to 90–95% on adding surfactant to the plasma samples prior to the analysis The relative standard deviation of the proposed clean-up procedure is 3 5% ($n = 6$) for both drugs measured at the 2 μg/ml level without using an internal standard The limit of determination with 100-μl injections is 0 10–0 15 μg/ml for ultraviolet detection and is seven times lower with electrochemical detection Teniposide was determined in

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patients' plasma and the results agreed well with those obtained by the conventional procedure involving manual liquid–liquid extraction prior to chromatographic analysis

INTRODUCTION

High-performance liquid chromatographic (HPLC) methods applied in bioanalysis usually require elaborate sample pretreatment, including deproteinization, removal of interferences and sample enrichment Traditionally, with lipophilic analytes, the method of choice is liquid–liquid extraction (LLE), followed by separation of the phases, evaporation of the organic layer to dryness and reconstitution of the residue in the mobile phase In recent years, there has been a trend to facilitate or to automate this time-consuming and sometimes cumbersome LLE procedure, e.g., by using robotics [1]

A different approach to achieving simplification is to change from sampling-processing-in-batch procedures to sample-processing-in-flow, which is much more easily automated. Usually, the latter involves solid-phase extraction (SPE) instead of LLE The use of SPE for sample clean-up and enrichment has been described extensively and has been applied both off-line ([2–5] and on-line [6–8] Many different applications and features of SPE have been reviewed [9,10]

The use of SPE and on-line HPLC provides direct injection of untreated samples. Special care has to be taken with the analysis of plasma or serum samples because of the presence of proteins The proteins tend to precipitate during the analysis and the system thus becomes clogged Moreover, the drugs to be analysed may be protein-bound and need to be released to permit detection Usually, the proteins are precipitated before analysis, by means of which, additionally, drugs are displaced from plasma components Several other methods have been reported for preventing interference of proteins, e.g., enzymatic protein hydrolysis [11] or dialysis [12] Direct injection of small volumes of plasma or serum samples using conventional reversed-phase HPLC (RP-HPLC) has also been described. Clogging of the system is prevented by avoiding high percentages of organic modifier in the mobile phase [13–16] or by working at elevated temperature [17]. More recently, direct plasma injection has been achieved by employing special column packings, such as internal surface reversed-phase support, to eliminate protein interferences [18–23] However, this approach is restricted because the range of available bonded phases is limited and only low concentrations of organic modifier are allowed. Finally, direct plasma or serum injection has been carried out successfully by using a micellar mobile phase in RP-HPLC (micellar liquid chromatography or MLC) [24–28] The micelles prevent the precipitation of proteins, but give only moderate chromatographic efficiency

Recently, the use of micellar clean-up prior to liquid chromatography has

been introduced [29,30] Sodium dodecyl sulphate (SDS) was added to the load mobile phase that was used to load untreated plasma samples on to a pre-column The proteins are solubilized by the SDS and washed out, whereas the analyte is retained. Next, the retained analyte and some residual endogenous compounds are eluted with the chromatographic mobile phase and analysed using conventional RP-HPLC

This paper describes an alternative surfactant-mediated clean-up procedure suitable for handling untreated plasma samples Addition of SDS to plasma samples before the analysis is demonstrated to be preferable and a micellar loading and/or washing phase is shown to be redundant The clean-up procedure has been automated and applied on-line with HPLC by using a programmable column-switching system The method has been optimized for the determination of teniposide in plasma and applied to monitoring therapeutic levels of teniposide in patient plasma.

EXPERIMENTAL

Chemicals and solutions

Methanol, sodium dihydrogenphosphate and sodium hydroxide, all of analytical-reagent grade, were purchased from Baker (Deventer, The Netherlands) Demineralized water was purified in a Milli-Q system (Millipore, Bedford, MA, U S A) and used to prepare buffer and standard solutions Buffer solutions were prepared freshly every week and filtered through a 0 2-μm filter (Type 11106; Sartorius, Gottingen, F R G) prior to use A 10 m*M* sodium phosphate buffer (pH 7 0) was used for the clean-up procedure and for the preparation of the chromatographic mobile phase SDS, obtained from Brocacef (Maarssen, The Netherlands), was used as received SDS solutions were prepared in sodium phosphate buffer (50 m*M*, pH 7) and filtered through a 0 2-μm filter (Type 11607, Sartorius). Etoposide (VP 16-213) and teniposide (VM 26) were kindly donated by Bristol Myers (Weesp, The Netherlands) Stock solutions of teniposide (1 0 and 0 1 μg/ml) and of etoposide (0 5 μg/ml) were prepared in methanol and stored at 4 °C Etoposide was used as the internal standard Standard working solutions of teniposide were prepared freshly by diluting the methanolic stock solutions with appropriate volumes of sodium phosphate buffer (10 m*M*, pH 7 0). Drug-free plasma was obtained from healthy volunteers

Plasma

Plasma was centrifuged prior to use at 3000 *g* for 10 min to obtain a clear supernatant, free from particulate matter.

Calibration standards Appropriate volumes of the methanolic stock solutions of teniposide were transferred into 1.5-ml polypropylene tubes. After evaporation of methanol under nitrogen, 450 μl of blank plasma, 50 μl of 380

m*M* SDS solution and 5 μl of the etoposide solution were added The spiked plasma samples were sonicated for 5 min

Patients' plasma To 450 μl of patient plasma, 50 μl of 380 m*M* SDS solution and 5 μl of the etoposide solution were added.

Apparatus

A diagram of the experimental set-up is shown in Fig. 1 The PROgrammable Multidimensional Injection System, PROMIS (Spark Holland, Emmen, The Netherlands), consisted of an autosampler, a Rheodyne injection valve (Type 7010) provided with a 100-μl sample loop and two six-port Rheodyne switching valves (Type 7010) Pump 1 (Model IPN8; Ismatec, Zurich, Switzerland, or Model M-45, Waters Assoc , Milford, MA, U S A) was used for loading the sample on to the clean-up column (CC). Pump 2 (TW 1515, Orlita Dosiertechnik, Giessen, F.R.G) was used to deliver the chromatographic mobile phase A Model 440 absorbance detector (Waters Assoc) (D_1) was used for monitoring the effluent from the analytical column (AC). With additional electrochemical detection (ED), a laboratory-made electrochemical detector cell [31] connected to a Metrohm 641 VA potentiostat was used (D_2). Signals were recorded on a Kipp recorder (Model BD 41 or BD 8).

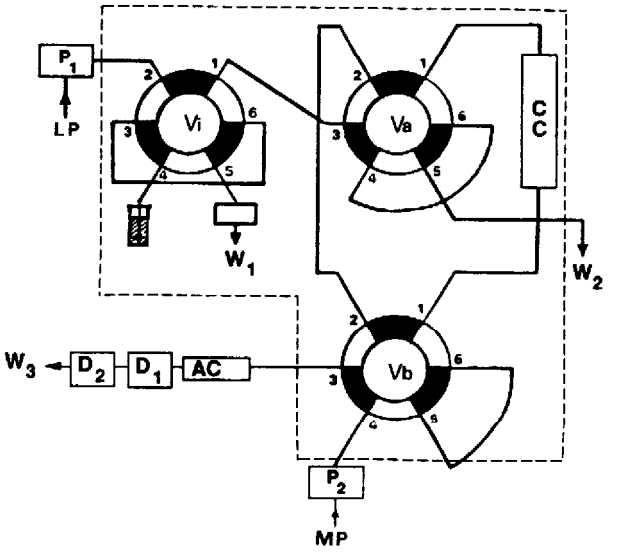

Fig 1 Schematic diagram of the experimental set-up The parts within the dotted box belong to the PROMIS, LP, loading phase, Vi, injection valve, Va, Vb, switching valves, CC, clean-up column, AC, analytical column, MP, chromatographic mobile phase, P_1, P_2, pumps, D_1, UV detector, D_2, electrochemical detector, W_1, W_2, W_3, waste

Conditions and procedure

A 10 mm×2 1 mm I D preconcentration column (Chromsep C_{18}, particle diameter = 40 μm; Chrompack, Middelburg, The Netherlands) was used for plasma clean-up. The samples were loaded with 10 m*M* sodium phosphate buffer (pH 7 0) at a flow-rate of 0 4 ml/min The loaded clean-up column was rinsed with the same buffer solution to wash out the solubilized proteins and other water-soluble interfering plasma components Next, the clean-up column was switched on-line with the RP-HPLC system. The retained components were eluted and separated by using the chromatographic mobile phase [10 m*M* sodium phosphate buffer (pH 7)–methanol (45 55, w/w)] at a flow-rate of 1 0 ml/min on a 300 mm×4 6 mm I.D analytical column (μBondapack Phenyl, particle diameter = 10 μm, Waters Assoc) UV detection at 254 nm was used during the development of the method The linearity and detection limit of the optimized method were measured with both UV detection and ED (+500 mV vs Ag/AgCl) Teniposide in patients' plasma was determined using ED and/or UV detection

Time scheme

Fig 2 shows the time schedule of the concurrent sample clean-up and HPLC analysis The outer ring represents the clean-up time cycle consisting of loading the sample on to the clean-up column (t = 0–7 5 min), eluting the retained components on to the analytical column (t = 7 5–8 5 min), regenerating the

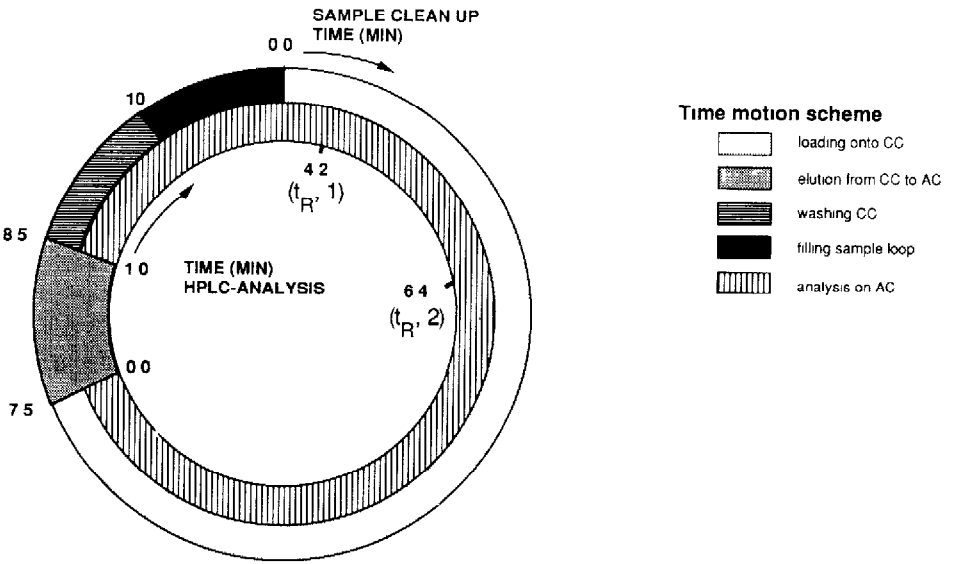

Fig 2 Time schedule of the concurrent sample clean-up (outer ring) and the chromatographic analysis (inner ring) with the retention time, t_R, for etoposide (1) and teniposide (2) CC = clean-up column, AC = analytical column

clean-up column (t=8 5–10 min) and filling the sample loop with the next sample (t=10–11 5 min) Then the cycle restarts at t=0 min The inner ring represents the HPLC analysis time cycle, consisting of injection of the retained components on to the analytical column (t=0–1 min) followed by separation The retention times, t_R, of etoposide and teniposide, 4 2 and 6 4 min, respectively, are indicated in the inner ring

RESULTS AND DISCUSSION

Optimization of the surfactant-mediated clean-up procedure

SDS added to the load mobile phase In two different micellar clean-up studies reported previously [29,30], SDS was used by adding it to the load mobile phase Initially, a similar approach was intended in this study The SDS concentration must be optimized, as both the retention behaviour and the solubilizing potency are influenced by the concentration of SDS in the load mobile phase Therefore, the breakthrough volumes of etoposide and teniposide on the CC were determined at several SDS concentrations The results for etoposide are summarized in Table I The breakthrough volumes for teniposide were slightly higher As expected [28], the retention, and consequently the breakthrough volume, decreases with increasing SDS concentration Hence, high SDS concentrations in the loading phase should be avoided On the other hand, the SDS concentration has to be sufficient to solubilize the proteins [32] A loading phase containing 20 m*M* SDS, which is about twice the critical micelle concentration (CMC) of SDS in aqueous solutions, was chosen for the initially intended micellar clean-up procedure

After loading the plasma samples on to the CC with the 20 m*M* SDS solution, the CC was rinsed with aqueous buffer and then switched on-line with the analytical column The clean-up procedure proved to be successful for eliminating the interference of plasma proteins The recycling capacity of the CC, which was defined as the maximum number of plasma injections on to a single CC without an increase in the back-pressure, was 35 for 100-μl injections Unfortunately, the recovery appeared to be only 30 and 8% for etoposide and

TABLE I

RELATION BETWEEN SDS CONCENTRATION IN THE LOADING PHASE AND BREAKTHROUGH VOLUME FOR ETOPOSIDE

SDS (m*M*)	Breakthrough volume (μl)
5	> 1000
10	500
20	400
50	220

teniposide, respectively These very low recoveries may be explained by the very high drug–protein binding shown by etoposide and teniposide [33] Although SDS, in addition to protein solubilization, also effects displacement of drugs from the plasma components [34,35], 20 m*M* SDS in the loading phase seems to be insufficient to release these highly protein-bound drugs.

SDS added to the plasma samples To obtain more effective drug releases, SDS was added directly to the plasma at concentrations up to 20 m*M*, which should be sufficient to achieve quantitative drug replacement [35] Too strong dilution was prevented by adding only 50 μl of 200 m*M* SDS to 450 μl of plasma The recovery increased to 95–98% for both components Unfortunately, this approach proved to cause problems with respect to the recycling capacity of the CC, which decreased to about 10 or 15 for 100-μl injections After few injections, the back-pressure in the CC began to increase, leading to deteriorated peak shapes and finally to column clogging

Although the CC could easily be emptied and dry-repacked with new stationary phase, several attempts were made to prevent pressure build-up For instance, analytical-reagent grade SDS was used, or the inlet surface of the CC was enlarged, or back-flush instead of forward-flush elution was performed. None of these modifications resulted in any improvement. However, on closer observation, it appeared that the plasma samples gradually became turbid after adding SDS to the plasma. Apparently, 20 m*M* SDS when added to plasma is not able to prevent (protein) precipitation in plasma samples, whereas 20 m*M* SDS when added to the aqueous loading phase and injection of SDS-free plasma samples was found to be satisfactory This difference may be explained by the dynamic nature of the micellar loading procedure involving both continuous supply of SDS and continuous removal of the proteins Second, the solubilizing properties of SDS in aqueous solution might be different from those in plasma [32]

The influence of the amount of SDS on the turbidity of plasma was investigated by varying the SDS concentration between 10 and 50 m*M* Surprisingly, at 20 m*M* SDS the turbidity turned out to be strongest At 30 m*M* the plasma was less turbid and at 40 m*M* it remained clear for several hours A concentration of 38 m*M* SDS was used for all further experiments with plasma.

Final clean-up procedure The use of SDS serves to solubilize the plasma proteins and to release the drug from the plasma proteins. Because both functions appeared to be fulfilled by adding 38 m*M* SDS to the plasma samples, it seemed that the use of a micellar load mobile phase can be omitted Therefore, the clean-up procedure could be simplified by combining the separate loading and rinsing step into a single step. Plasma samples containing 38 m*M* SDS were loaded on to the CC with phosphate buffer (10 m*M*, pH 7) and the CC was rinsed with the same buffer solution. No breakthrough was observed using 3 0 ml of the aqueous buffer, while the breakthrough volume with micellar mobile load phase (20 m*M* SDS) was only 0 4 ml (Table I). Apparently, the

breakthrough volume increases markedly when SDS is added to plasma instead of to the loading phase This phenomenon may be explained by depletion of micelles in plasma owing to binding of SDS to the proteins by which the free SDS concentration is lowered The recycling capacity of the CC using this final clean-up procedure proved to be at least 100 for 100-μl plasma injections.

The chromatogram obtained after applying this final clean-up procedure prior to HPLC assay is shown in Fig 3 Only a few endogenous plasma components are observed (Fig. 3a), which are well separated from etoposide and teniposide (Fig. 3b).

Finally, the preconcentration potency of the proposed final clean-up procedure was investigated. A standard solution of teniposide (10 μg/ml) in plasma which contained 38 mM SDS was prepared. The injection volume was increased from 100 μl to 2 ml in steps of 100 μl. The blank response did not increase significantly up to an injection volume of 2 ml A linear relationship (r=0 9997) between the injection volume and the peak area was observed. After five repeated injections [relative standard deviation (R.S D) = 2%] of 2 ml of plasma the CC has to be replaced because of pressure build-up. Hence, to achieve the maximum recycling capacity of the CC, the injection volume should be as low as possible On the other hand, the sensitivity of the method

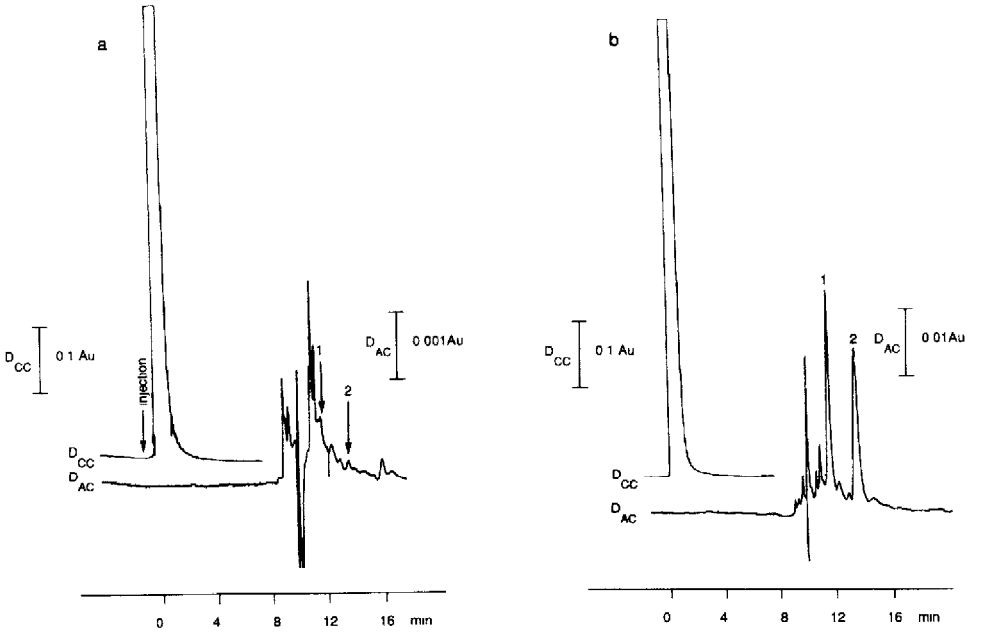

Fig 3 UV signals at 254 nm for the clean-up column effluent (D_{CC}) and of the analytical column effluent (D_{AC}) after the surfactant-mediated clean-up procedure (a) 100 μl of blank plasma, the usual positions of etoposide (1) and teniposide (2) are indicated by arrows, (b) 100 μl of spiked plasma, 10 μg/ml each of etoposide (1) and teniposide (2)

can easily be increased by increasing the injection volume. An injection volume of 100 μl was chosen throughout this study.

Recovery and reproducibility

The recovery from SDS-containing plasma and from SDS-containing buffer with respect to aqueous buffer (without SDS) was determined. Plasma was spiked with teniposide at levels ranging from 0 5 to 25 μg/ml and SDS was added to a final concentration of 38 m*M*. Standard solutions of teniposide with concentrations ranging from 0 5 to 25 μg/ml were prepared in buffer that contained 38 m*M* SDS and in aqueous buffer The prepared samples and standards were analysed according to the final clean-up procedure using the automated column-switching systems with UV detection Three calibration graphs were obtained with intercepts that did not differ significantly from zero The slopes were 1.04, 0.94 and 0.50 for aqueous buffer, plasma with 38 m*M* SDS and buffer with 38 m*M* SDS, respectively, corresponding to a mean recovery of 90% for plasma with 38 m*M* SDS and only 48% for buffer with 38 m*M* SDS. This low recovery from SDS-containing buffer may be caused by loss of teniposide due to breakthrough. This phenomenon indicates different behaviours of a similar concentration SDS in aqueous solution and in plasma In the latter the SDS was largely bound to the proteins, with the result that the free concentration was lowered [32]

The reproducibility of the final clean-up procedure was determined for UV measurements The R.S D ($n=6$) without using an internal standard was 3.5% at the 2 μg/ml level and 2.0% at the 10 μg/ml level for both drugs For teniposide the R.S.D. was also calculated considering etoposide as the internal standard The R S.D. was 3.0% at both levels These results indicate that the reproducibility of the proposed final clean-up procedure is not improved by using an internal standard.

Linearity and detection limit

Linearity and detection limits were determined for both UV and electrochemical detection for aqueous and plasma standards As evaluated above, for UV detection no internal standard is needed For ED, however, it is known that the sensitivity may decrease owing to contamination of the electrode surface and internal standardization is advisable. In the ED experiments, teniposide was measured using etoposide as an internal standard The results are summarized in Table II. The linearity is excellent for all calibrations. The limit of determination (LOD) is calculated at a signal-to-noise ratio of 3 UV detection resulted in an LOD of 150 and 100 ng/ml for teniposide and etoposide, respectively, Obviously, the LOD is lower for ED and proved to be 20 ng/ml for teniposide.

Patients' plasma

Some patients' plasma samples containing teniposide at levels between 5 and 15 μg/ml were measured by both UV and electrochemical detection. The

TABLE II

LINEARITY AND LIMIT OF DETERMINATION (LOD) FOR UV AND ELECTROCHEMICAL DETECTION

Sample		Slope	Intercept	r	LOD[a]
UV[b] (1-25 μg/ml)					
Aqueous	etoposide	2 08	0 11	0 9999	
	teniposide	1 04	0 16	0 9999	
Plasma	etoposide	1 89	−0 16	0 9999	100
	teniposide	0 94	−0 10	0 9999	150
ED[c] (0 25-25 μg/ml)					
Aqueous	etoposide	0 075	0 008	0 9998	
Plasma	teniposide	0 072	−0 013	0 9997	20

[a]Limit of determination (ng/ml) for plasma calculated at a signal-to-noise ratio of 3
[b]UV detection without using an internal standard
[c]Electrochemical detection using an internal standard (etoposide)

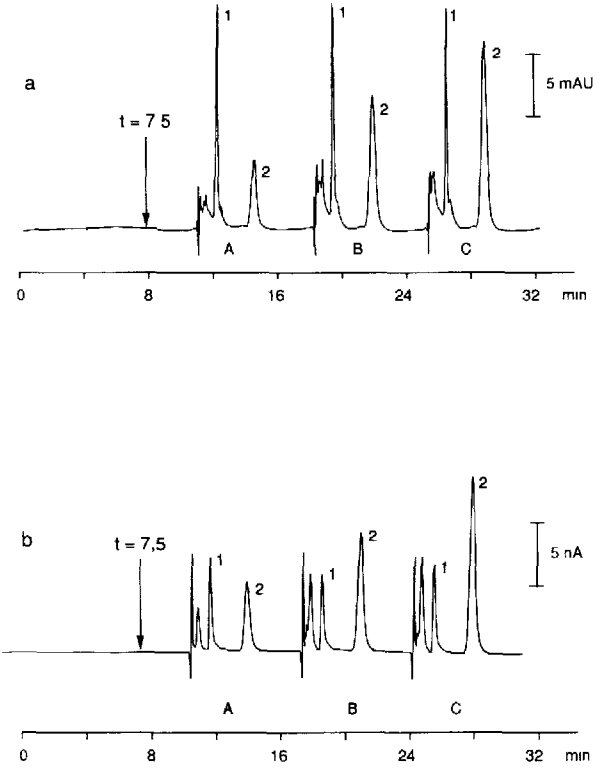

Fig 4 Chromatograms obtained after assay of patients' plasma containing teniposide (5 0, 10 6 and 15 6 μg/ml for A, B and C, respectively) (a) UV detection at 254 nm, (b) ED at +500 mV vs Ag/AgCl The peaks of the internal standard etoposide (1) and of teniposide (2) are indicated

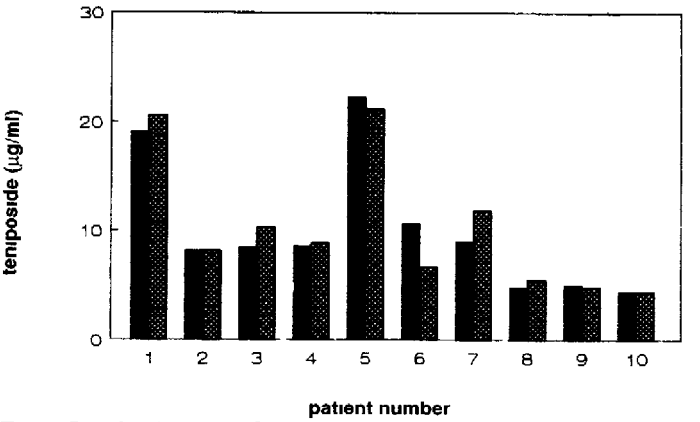

Fig 5 Levels of teniposide in patients' plasma measured by reversed-phase HPLC after manual liquid–liquid extraction (hatched boxes) or automatic surfactant-mediated clean-up (black boxes)

chromatograms of three different patients' plasma samples are shown in Fig 4 Because of the high levels of teniposide in these samples, ED is not required UV detection is satisfactory, despite some additional interfering components observed by UV compared with electrochemical detection

Comparison with conventional LLE clean-up

The developed method of analysis was applied to the determination of teniposide in patients' plasma. Each plasma sample was treated according to both the conventional manual LLE procedure [31] and the proposed automated surfactant-mediated clean-up (SMC) procedure The samples were analysed under identical chromatographic conditions and ED was applied The results, which are shown in Fig 5, demonstrate that only slight differences occurred The correlation proved to be 0 953 The data were evaluated by the statistical *t*-test [36] at the 95% confidence level The differences between the two methods were not significant

The major advantage of the proposed SMC approach is that the sample processing can be fully automated. Additionally, as the method involves concurrent plasma sample clean-up and HPLC analysis, the total time of analysis decreases Approximately five samples per hour, including sample pretreatment, could be performed, whereas the conventional approach allowed the same sample throughput, but excluding the time-consuming manual sample pretreatment

CONCLUSIONS

The automated SMC procedure with direct plasma injection proved to be a good alternative to the fairly laborious and time-consuming manual LLE pro-

cedures. By using the column-switching technique, the SMC procedure can easily be applied on-line with RP-HPLC The proposed procedure has been demonstrated to be suitable for the determination of etoposide or teniposide in plasma. However, by adjusting the chromatographic conditions, e g , the dimensions of the CC, or the stationary phase in the CC, or the analytical separation conditions, the SMC–HPLC method might be suitable for other neutral drugs

The SMC procedure allows injection volumes of up to 2 ml, providing an on-line sample enrichment step for increasing the LOD Further, unlike in micellar chromatography, the efficiency of the analytical column is not adversely affected by the SDS in the proposed SMC–HPLC method

In contrast to other studies [29,30], in this study the SDS is added to the plasma samples and an aqueous buffer is used as the load mobile phase This approach proved to increase the recovery of highly protein-bound drugs dramatically and to permit simplification of the clean-up procedure because two separate steps of sample loading on to the CC and rinsing the CC are combined in a single step The concurrent sample clean-up and chromatographic analysis allows a sample throughput of 5 5 samples per hour

The recycling capacity of the CC was increased substantially, from about 15 to at least 100 for 100-μl injection volumes, when 38 m*M* instead of 20 m*M* SDS was added to the plasma For the assay of etoposide and teniposide, 38 m*M* SDS was found to be optimal because it (a) effects satisfactory protein solubilization, (b) leads to high recoveries and (c) maintains sufficient retention on the CC

The procedure can be used with either UV detection or ED ED is necessary for relatively low levels of teniposide in plasma, e g , for pharmacokinetic studies For monitoring therapeutic levels (0 1–50 μg/ml), UV detection is satisfactory The reproducibility of the proposed procedure is not increased by the use of an internal standard However, when ED is applied the use of an internal standard is advisable

The procedure can be used in principle for sample clean-up in flow-injection assay for etoposide and teniposide [37] However, the relatively high blank response still prevents the convenient combination of SMC with conventional flow-injection methods Work is in progress to combine the proposed SMC procedure with a more selective flow-injection method using multi-channel detection and multivariate data analysis

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