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Comparative study on the determination of the anti-neoplastic drug teniposide in plasma using micellar liquid chromatography and surfactant-mediated plasma clean-up

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ABSTRACT

The potential of micellar liquid chromatography and of an on-line surfactant-mediated sample clean-up, which involves column-switching prior to conventional reversed-phase high-performance liquid chromatography, has been evaluated for the determination of the anti-neoplastic drug teniposide in plasma by using electrochemical detection. A major advantage of surfactant-mediated techniques is that they allow fully automated processing of plasma samples, because protein precipitation is prevented by the addition of the surfactant sodium dodecylsulphate. With the automated column-switching technique, a degree of sample enrichment and of selectivity can be attained, which is similar to that for the conventional procedure which, however, involves a labour-intensive off-line isolation of teniposide, using liquid–liquid extraction prior to chromatography. An inherent drawback of automated micellar liquid chromatography is that no sample clean-up or preconcentration can be carried out, which results in only a moderate detection limit and selectivity. The linearity, reproducibility and recovery of the surfactant-mediated techniques are similar to those of the conventional procedure. Based on the presented results, it was concluded that the surfactant-mediated column-switching technique is a highly attractive sample enrichment technique with respect to simplicity, speed and cost.

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INTRODUCTION

Drug monitoring is often carried out in plasma samples. Unfortunately, the injection of untreated plasma samples into reversed-phase high-performance liquid chromatographic (RP-HPLC) systems is prevented by the presence of plasma proteins. These tend to precipitate in aqueous–organic mobile phases, which causes a rapid build-up of back-pressure. Therefore, traditionally, plasma samples are deproteinized prior to HPLC analysis by treatment with organic solvents or strong acids, by ultrafiltration, or by extracting the analyte into an organic solvent [1,2]. Disadvantages of deproteination are that it can cause problems with respect to the recovery and reliability of the analysis, and that it will complicate the automated drug monitoring in plasma. More recently, the fully automated on-line dialysis by using the ASTED autosampler is gaining more popularity for the clean-up of samples [2]. Despite its potential, on-line dialysis is still not applied in common HPLC set-ups.

It is, therefore, highly advantageous if deproteination can be circumvented. Several approaches for direct plasma injection (DPI) into HPLC systems have been reported [3,4]. First, if the sample volume does not exceed a few microlitres, repetitive sample injection in conventional RP-HPLC systems is possible [5]; obviously, the attainable detection limit will not be too low. Secondly, special internal-surface reversed-phase [6,7] or shielded hydrophobic-phase columns [8] can be used. However, these columns do not allow high percentages (over 15–30%) of an organic modifier in the mobile phase. Thirdly, DPI can be applied to RP-HPLC systems, through substitution of the organic modifier by anionic or non-ionic micellar mobile phases, in which the proteins do not precipitate [9–12]. However, micellar liquid chromatography (MLC) still is not widely used in routine analysis, which is at least partly due to its only moderate efficiency compared with conventional RP-HPCL systems [13]. Finally, DPI can be carried out by using a column-switching technique in which the proteins are removed from the sample prior to HPLC analysis. Traditionally, plasma is injected into a pre-column by using an aqueous carrier phase, which allows lipophilic analytes to be retained on the pre-column, while the proteins are flushed to waste [14–16]. Next, the pre-column is switched on-line with a conventional RP-HPLC system. The potential for trace enrichment is excellent and low detection limits can be obtained [3,4]. However, the recovery may cause problems with polar or highly protein-bound drugs [4,17]. More recently, aqueous anionic micellar solutions of, *e.g.*, sodium dodecylsulphate (SDS), have been used as a carrier phase [18,19]. The limited results do not allow a thorough evaluation of this approach, but it seems that high protein binding still prevents a satisfactory recovery of drugs from plasma samples [18].

However, in our previous study [20] it was demonstrated that, if SDS is added directly to the plasma sample instead of merely being used as a micellar carrier phase, the recovery of the highly protein-bound (greater than 99.6% [21]) anti-neoplastic drug teniposide is markedly increased, *i.e.* from a mere 8% with surfactant-free plasma to 90% [20].

The aim of the present study was to investigate MLC and the described column-switching technique for drug monitoring in plasma, with respect to practicality and analytical performance. For a proper evaluation of these aspects, the results were compared with those of a conventional assay which involves a labour-intensive off-line isolation of the drug from plasma using liquid–liquid extraction [21]. To our knowledge no such evaluation of the novel surfactant-mediated techniques has been carried out before.

The determination of teniposide in plasma was selected as a model system for this study. Teniposide has a therapeutic range of 0.1–50 μl/ml in plasma, and is often detected by UV or electrochemical detection [22] after HPLC separation. In the present study the latter detection mode was used.

EXPERIMENTAL

Chemicals and solutions

Demineralized water was used throughout. Sodium dodecylsulphate, 1,2-dichloroethane, methanol, *n*-propanol and 2,6-dimethoxyphenol were from Merck (Darmstadt, Germany), and were used as received. Teniposide and etoposide were a gift from Bristol Myers Nederland (Weesp, The Netherlands). Stock solutions were prepared of etoposide and teniposide (200 μg/ml) in methanol, and of 2,6-dimethoxyphenol (200 μg/ml) in water.

The micellar mobile phase used for method 1 (see below) was prepared by dissolving SDS (40 m*M*) and *n*-propanol (500 m*M*) in a 10 m*M* phosphate buffer (pH 7.0), followed by filtration through a 0.2-μm filter (Type 11106; Sartorius, Göttingen, Germany) and ultrasonic deaeration. The mobile phase used for methods 2 and 3 was methanol–10 m*M* phosphate buffer (pH 7.0) (55:45, w/w), which was filtered and deaerated ultrasonically prior to use.

Plasma was obtained from healthy volunteers or from patients treated with teniposide.

Micellar chromatography (method 1)

Sample preparation. Plasma was centrifuged at 3000 *g* for 10 min to remove particulate matter. To a volume of 900 μl of patient or spiked plasma, 50 μl of an aqueous 800 m*M* SDS solution and, typically, 50 μl of the 2,6-dimethoxyphenol solution (internal standard) were added.

Chromatography. A 20-μl volume of plasma sample was injected into an automated HPLC system, which consisted of a Spectroflow 400 pump (Kratos, Ramsey, NJ, U.S.A.), a Gilson 231 autosampler (Gilson, Villiers-le-Bel, France), a 10 mm × 2.1 mm I.D. pre-column (40-μm Chromsep C_{18}; Chrompack, Middelburg, The Netherlands) and a 100 mm × 2.1 mm I.D. analytical column (5-μm Chromspher C_{18}, Chrompack). The columns were thermostatted at 40°C. The effluent (1 ml/min) was monitored using a laboratory-made wall-jet electrochemical detector [23], which was connected to a Model 641 VA potentiostat (Metrohm, Herisau, Switzerland). The detector cell consisted of an EA 286/1 glassy carbon working electrode (Metrohm), a stainless-steel auxiliary electrode and a silver/silver chloride reference electrode. The applied potential was +500 mV. The glassy carbon electrode was cleaned daily by polishing with 0.3-μm Al_2O_3 powder. The chromatograms were recorded on a Model DB 41 recorder (Kipp & Zonen, Delft, The Netherlands) or a Model 4270 integrator (Spectra-Physics, Santa Clara, CA, U.S.A.).

Surfactant-mediated sample clean-up (method 2)

Sample preparation. Plasma was centrifuged at 3000 *g* for 10 min to remove particulate matter. To 900 μl of spiked or patient plasma, 100 μl of an aqueous 380 m*M* SDS solution and, typically, 50 μl of the internal standard were added. The sample was then vortexed for 10 s.

Chromatography. The automated clean-up procedure has been described previously [20]. A 100-μl aliquot of the plasma sample was injected into a 10 mm × 2.1 mm I.D. pre-column (40-μm Chromsep C_{18}; Chrompack) using a Promis autosampler (Spark, Emmen, The Netherlands). The pre-column was rinsed with 3 ml of a 10 m*M* phosphate buffer (pH 7.0) and subsequently switched on-line with the HPLC system, allowing the analytes to elute at a flow-rate of 0.7 ml/min into a 300 mm × 4.6 mm I.D. analytical column (10-μm Bondapak Phenyl; Millipore-Waters, Milford, MA, U.S.A.). Electrochemical detection was carried out at +500 m*V*, as described above.

Reference procedure (method 3)

Sample preparation. The sample preparation procedure has been described previously [21]. To 1.0 ml patient or spiked plasma, 50 μl of the internal standard (etoposide) were added. After vortexing, 2 ml of dichloroethane were added. The mixture was shaken thoroughly for 1 min and centrifuged at 3000 *g* for 5 min. About 1.5 ml of the dichloroethane layer were pipetted into a polypropylene reaction vial (Type 3810; Eppendorf, Hamburg, Germany), and dichloroethane was evaporated at room temperature under a stream of dry nitrogen. The residue was reconstituted in 150 μl of methanol–water (70:30, v/v), by using sonication for 6 min, and subsequently transferred to a stoppered sample vial.

Chromatography. A 10-μl volume of the sample was injected into an automated HPLC system that consisted of a Spectroflow 400 pump (Kratos), a WISP autosampler (Millipore-Waters), a laboratory-made 10 mm × 4.6 mm I.D. pre-column, filled with 10-μm LiChrosorb C_{18} (Merck) and a 100 mm × 4.6 mm I.D. analytical column (10-μm Phenyl Novapak, Millipore-Waters). The effluent (0.7 ml/min) was monitored by electrochemical detection at +500 mV, as described above.

Statistical analysis

The three analytical procedures were statistically compared according to the method of maximum total error, described by Wolters and Kateman [24]. This method allows the calculation of the most probable maximum difference between the determined and the true concentrations of teniposide in plasma. For this, four series of calibration curves were separately prepared for each procedure, by spiking blank plasma with 0–20 μg/ml of teniposide, at intervals of 2.5 μg/ml. These samples were analysed. The spiked concentrations were taken as the true teniposide concentrations.

RESULTS AND DISCUSSION

Micellar chromatography (method 1)

Optimization of micellar mobile phase. The main reason for investigating the utility of MLC for the determination of drugs in plasma is that this technique offers the opportunity for direct plasma injection in reversed-phase chromatography, whereas this is virtually impossible with conventional RP-HPLC systems [9–12]. In MLC the capacity factor, k', of the analyte is significantly influenced by the concentration of the micelles. In the present study, k' was optimized by adjusting the SDS concentration in a 10 m*M* phosphate buffer (pH 7.0), which contained 500 m*M* *n*-propanol to improve the column efficiency [25,26]. It appeared that at low SDS concentration, *i.e.* lower than 20 m*M*, the back-pressure in the pre-column increased steeply after a few injections, owing to protein precipitation. At higher SDS concentrations, *i.e.* larger than 80 m*M*, the internal standard was insufficiently separated from the endogeneous plasma compounds. A micellar mobile phase containing 40 m*M* SDS and 500 m*M* *n*-propanol was selected for all further experiments.

Next to the addition of an organic modifier, increasing the column temperature can help to improve the moderate efficiency of MLC [26,27]. Although at elevated temperatures back-pressure may increase in the MLC system because of protein precipitation, this was not observed up to 55°C. However, because the column efficiency did not improve above 40°C, the latter temperature was selected for all further work.

In the optimized MLC set-up, the plate number for teniposide in the analytical column is *ca.* 1200 (5-μm particles, $k' = 10$), whereas with conventional RP-HPLC it is 1700 (10-μm particles, $k' = 8$).

Optimization of sample treatment. In previous studies, untreated plasma was directly injected into the MLC system. However, this may cause a rather broad elution profile of the plasma proteins, *viz.* of up to 20 min [11,12]. In the present study no such broad plasma peak was observed: the detector response returned to the baseline within 6 min. However, the back-pressure in the column increased, an only a few plasma samples could be injected. The back-pressure build-up can be explained as follows. In a previous study it has been demonstrated in batch experiments that plasma proteins are sufficiently solubilized only if over *ca.* 35 m*M* SDS is present [20]. Between the critical micelle concentration (8.3 m*M*) and 35 m*M*, protein precipitation is observed [20]. This may be due to an insufficient shielding of the exposed hydrophobic domains of the denatured proteins [29,30]. In other words, in batch protein solubilization is observed only above a certain threshold concentration of the surfactant. As regards the direct injection of untreated plasma in the case of MLC, attainment of the threshold SDS concentration in the vicinity of the proteins in the surfactant-deprived sample plug will be markedly affected by the mixing kinetics in the chromatographic system. During mixing, *i.e.* at intermediate surfactant concentrations, protein precipication may well take place.

The above effect may also be an explanation for the observed peak splitting of highly protein-bound drugs after injection of untreated plasma [9]. A complete release of drugs from proteins takes place only at SDS concentrations above 10–20 m*M* [20,27,30,31].

If SDS is added to the plasma sample prior to injection, these problems may well be prevented. Based on our earlier experience [20], SDS was added to the plasma sample to yield a final concentration of 40 m*M*. As a result, there was no steep back-pressure build-up, and the number of injections could be increased from a mere 6 to 80 on a single column. Additional advantages were that the peak shapes improved somewhat, and that the recovery of teniposide became slightly better, *i.e.* 97 ± 2% (n = 6) instead of 89 ± 2% (n = 3), both compared with the injection of teniposide in buffer. The modest improvement of the recovery in MLC is in marked contrast to the marked improvement observed in surfactant-mediated clean-up (see below) [20]. This difference can be explained by the fact that if untreated plasma is used in the column-switching technique, the highly protein-bound drug passes through the pre-column with the proteins, and is virtually completely lost.

Optimization of the electrochemical detector. The use of an electrochemical detector in combination with MLC has hardly been reported [32]; this was therefore studied in some detail. Fig. 1 shows the hydrodynamic voltammogram for teniposide in the present micellar mobile phase, which is essentially similar to that obtained in methanol–aqueous buffer eluents [21]. With freshly polished electrodes the signal-to-noise level at the applied potential was *ca.* five-fold lower than that in methanol aqueous buffer mixtures; this was caused by a higher detector noise, possibly because of the micro-heterogeneity of the micellar mobile phase or of the presence of small amounts of electroactive contaminants. An electrode potential of + 500 mV was selected for all further experiments, because at this potential a maximal signal-to-noise level was obtained and, furthermore, because interfering peaks from plasma appeared in the chromatogram on applying higher potentials.

It proved that the sensitivity of the electrochemical detector decreased in the long run in a similar manner as the conventional RP-HPLC system: that is, *ca.*

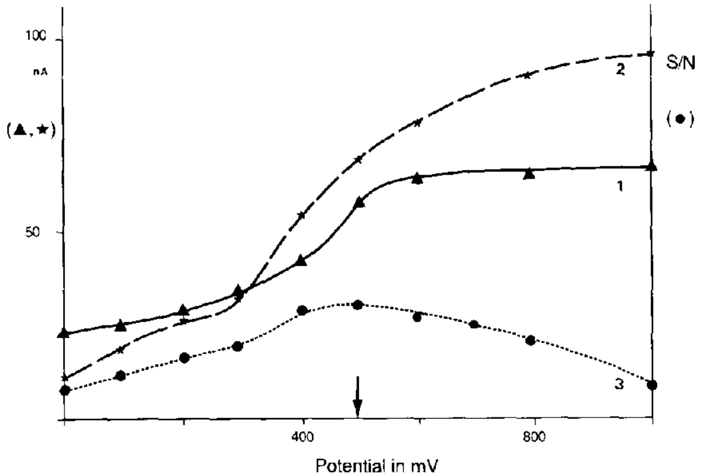

Fig. 1. Hydrodynamic voltammogram in aqueous–organic and micellar mobile phase, after repetitive injections of 200 ng teniposide: (1) 40 m*M* SDS in a 10 m*M* phosphate buffer with 500 m*M* *n*-propanol; (2) methanol–10 m*M* phosphate buffer (55:45, w/w); (3) signal-to-noise level for curve 1 (arbitrary units).

40% in 16 h [21]. Therefore, the use of an internal standard is advisable. 2,6-Dimethoxyphenol was selected for this purpose instead of etoposide, which co-eluted with a plasma constituent. Fig. 2A shows the chromatogram for teniposide in plasma at a concentration of 1.5 μg/ml, obtained with the optimized chromatographic system; Fig. 2B shows the chromatogram of blank plasma. Teniposide and 2,6-dimethoxyphenol are both nicely separated from the injection peak.

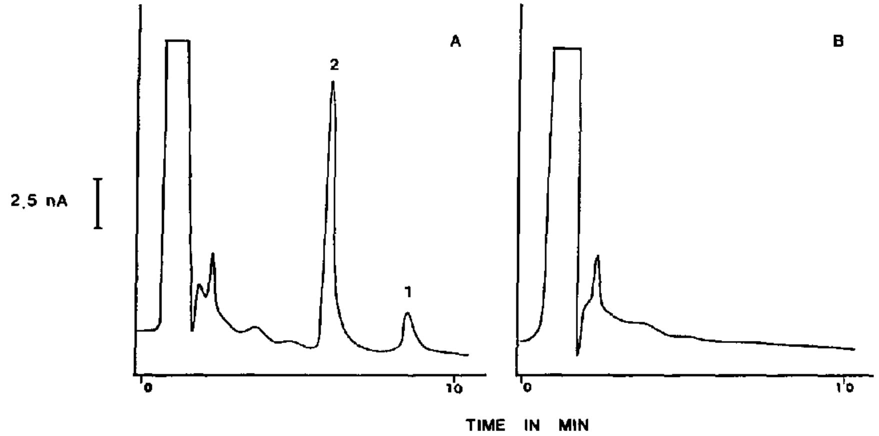

Fig. 2. Chromatograms of (A) plasma spiked with 1.5 μg/ml teniposide and (B) blank plasma, obtained with micellar liquid chromatography. Peaks: 1 = teniposide; 2 = 2,6-dimethoxyphenol (internal standard; 10 μg/ml). Conditions: see Experimental.

Surfactant-mediated sample clean-up (method 2)

The determination of teniposide in plasma using the surfactant-mediated sample clean-up has been discussed previously [20]. Here, a summary of the main features of the technique is given. First, with the column-switching procedure, 40 m*M* SDS is directly added to the plasma sample to solubilize the proteins and to release the drug from the proteins prior to sample-processing in the pre-column. The subsequent column-switching step allows an automated and efficient removal of proteins from the sample prior to RP-HPLC. It should be noted that this approach is in marked contrast to previously described procedures, in which the micelles were merely used as a carrier phase [18,19]. Compared with these procedures, our approach resulted in a significant increase of the recovery of teniposide, *i.e.*, from a mere 8% to *ca.* 90% (for explanation, see previous section). In addition, the number of injections into a single clean-up column could be increased from 30 to *ca.* 150. Secondly, the automated column-switching technique allows the concentration of the analyte from, for example, 1 ml of plasma. Such a sample volume is typically applied to disposable solid-phase cartridges, which are often more expensive and labour-intensive in use. Therefore, surfactant-mediated sample clean-up can be highly competitive for drug monitoring, as regards the simplicity and cost of the sample pretreatment. Finally, because the sample clean-up and HPLC are arranged in a concurrent mode, an acceptable sample throughput of *ca.* eight samples per hour can be realized.

Typical chromatograms obtained with the column-switching procedure for spiked and blank plasma are shown in Fig. 3A and B, respectively. The figures

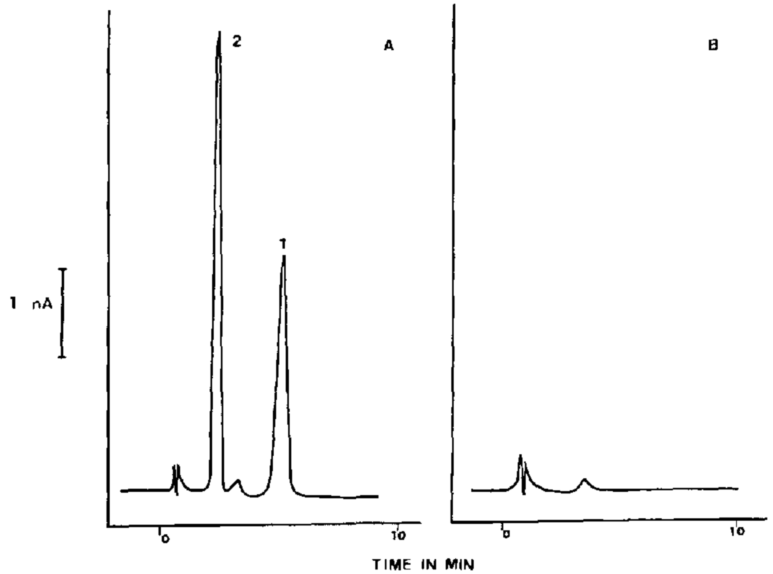

Fig. 3. Chromatograms of (A) plasma spiked with 2.5 μg/ml teniposide and (B) blank plasma, obtained with surfactant-mediated clean-up of plasma. Peaks: 1 = teniposide, 2 = etoposide (internal standard 5 μg/ml). Conditions: see Experimental.

demonstrate that only small amounts of other plasma compounds are observed in the chromatogram, despite the relatively simple sample manual pretreatment.

Reference procedure (method 3)

The conventional HPLC determination of teniposide in plasma has previously been described in more detail [21]. Prior to HPLC, teniposide is extracted from plasma using liquid–liquid extraction. The extraction procedure has a high recovery of 97% and allows the selective isolation of teniposide and internal standard [21]. After separation and subsequent evaporation of the organic layer, the drug is reconstituted in a much smaller volume, thereby allowing significant analyte enrichment. The major drawback of the sample preparation is that it is labour-intensive and is not easy to automate.

Fig. 4A shows a typical chromatogram of teniposide in plasma, and Fig. 4B of blank plasma. Teniposide and the internal standard are well separated from other plasma constituents.

Comparison of procedures

Analytical performance. Table I gives a compilation of several analytical parameters, which are discussed below. The table shows that, when using an internal standard, the repeatability of the three procedures is comparable and satisfactory. The recovery of teniposide is satisfactory in all procedures, and the detection limits of the reference and column-switching procedures are superior to

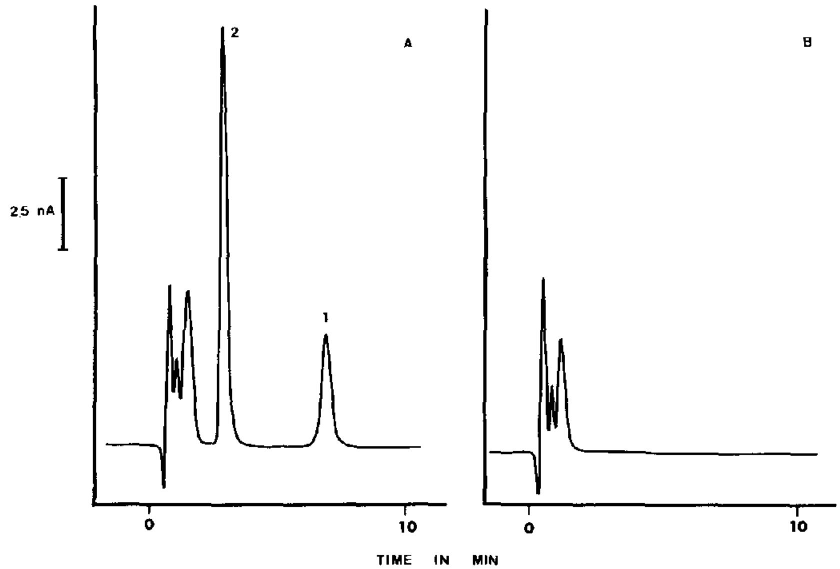

Fig. 4. Chromatograms of (A) plasma spiked with 1.5 μg/ml teniposide and (B) blank plasma, obtained with the reference procedure. Peaks: 1 = teniposide; 2 = etoposide (internal standard 5 μg/ml). Conditions: see Experimental.

TABLE I

COMPARISON OF THE ANALYTICAL PERFORMANCE OF THE MLC (METHOD 1), SMC (METHOD 2) AND REFERENCE (METHOD 3) PROCEDURES FOR THE DETERMINATION OF TENIPOSIDE IN PLASMA

Parameter	Method 1	Method 2	Method 3
Recovery (%)	97	90	96
Calibration curve			
Correlation (0–20 μg/ml)	0.998	0.999	0.999
Repeatability at 1 μg/ml (%; $n=6$)	3.6	3.0	2.9
Detection limit (ng/ml)	500	10	10
Manual handling time (min)	0.5	0.5	2
Sample throughput per day	150	180	100
Sample throughput per column	80	150	>1000
Cost (ECU)[a]	0.1	0.1	0.4

[a] Value per sample in European Currency Units.

that of MLC, owing to the concentration step prior to analysis. MLC seems, therefore, in principle less suited for trace analysis. This conclusion is supported by the examples in the literature [9–12]. The linearity of the calibration curves is fully satisfactory for all procedures in the teniposide concentration range determined. The selectivities of the three techniques for the determination of teniposide in plasma seem to be similar (*cf.* Figs. 2–4), despite the differences in the sample clean-up prior to analysis.

The performance of the procedures was statistically evaluated according to the method of maximum total error [24]. This approach allows the calculation of the most probable maximum difference between the measured and true values of the teniposide concentration in plasma and, furthermore, to discern the contributions of the random and of the systematic error in the total error of a given procedure. That is, the method provides a fair impression of the reliability of the experimental procedure. The results are presented in Fig. 5. The area between the two dashed lines represents the maximum experimental deviation from the true concentration with a 90% probability level. The solid line represents the most probable maximum difference.

Fig. 5 demonstrates that for all procedures (i) the most probable maximal random error is lower than 1 μl/ml, and (ii) the contribution of the systematic error is very similar for all three procedures. As regards the small differences between the systematic and random error for the procedures, this may well be related to the fact that the samples had to be spiked and prepared separately for the individual procedures. It would have been more appropriate to spike a single plasma sample for each teniposide concentration, and use aliquots for each of the

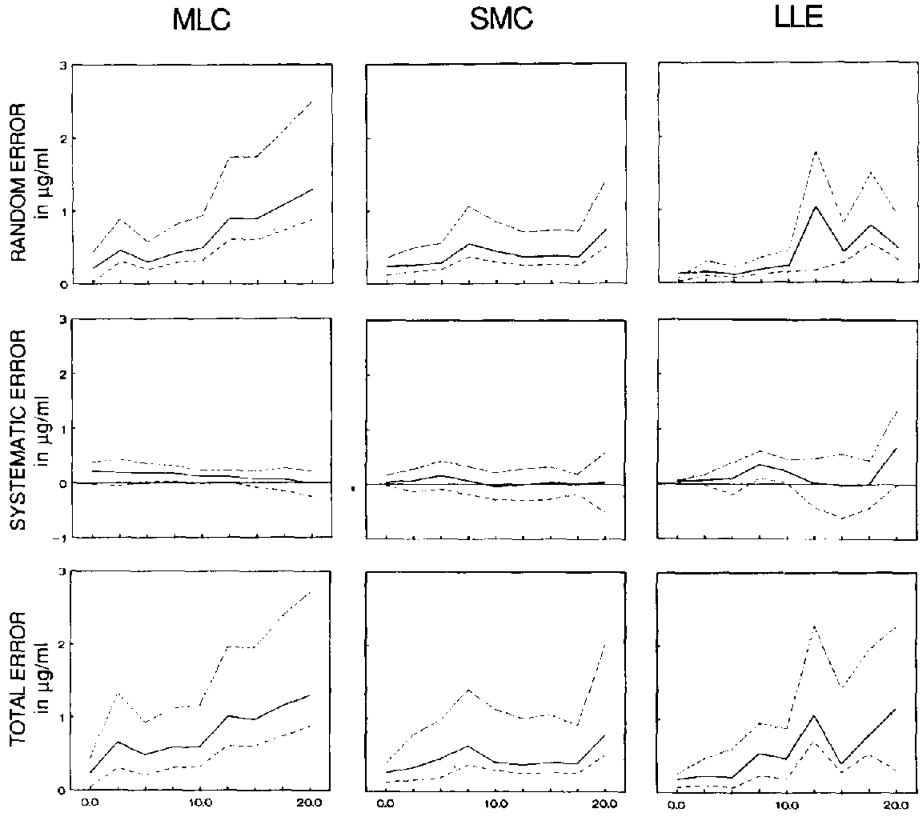

Fig. 5. Plots of the accuracy (top), precision (centre) and reliability (bottom) of the MLC, SMC and reference procedure, respectively, as a function of the teniposide concentration (µg/ml), obtained with the method of maximal total error [23]. The solid line represents the most probable maximum difference between the determined and true teniposide concentrations in plasma. The dashed lines show the limits of the 90% probability interval of the maximum difference between the determined and the true teniposide concentrations in plasma. In the case of random and systematic errors, the curves represent the contribution of these errors to the total error.

three procedures. However, it turned out that spiking of a plasma sample with such a volume caused problems with regard to the homogeneity of the sample. Nonetheless, from Fig. 5 it can safely be concluded that, with respect to the reliability, accuracy and precision of the procedures, no technique is superior within the concentration range tested.

Ten patient samples were simultaneously subjected to the three procedures. The drug levels are shown in Fig. 6. It can be seen that for each sample the three experimental values are close together; that is, the results of the MLC and the clean-up procedure do not significantly differ (Student's t-test, 95% confidence level [33]) from those of the reference procedure.

Practicality. Next to the analytical performance, aspects such as cost, speed

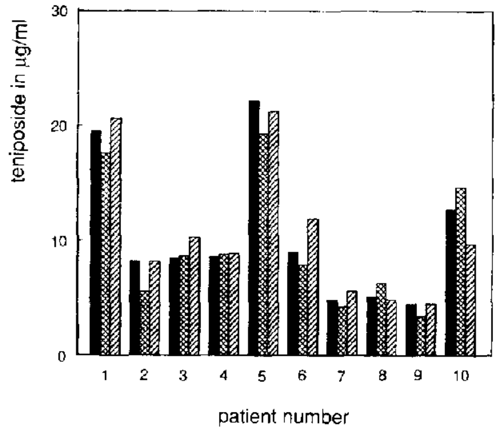

Fig. 6. Comparison of the determination of the teniposide concentration in ten patient samples with the reference (full bars), MLC (double-hatched bars) and SMC (hatched bars) methods.

and robustness of a method are selection criteria that should be considered.

An important selection criterion is the manual sample handling that is involved in the procedure. For the reference procedure, a skilled technician is occupied almost full-time with the labour-intensive off-line sample preparation. With both surfactant-mediated procedures, the manual preparation time can be fourfold reduced. As for the costs involved (disposables, chemicals and labour), the surfactant-mediated procedures are about four times cheaper than the reference procedure, mainly because of the reduced labour cost.

Comparison of the robustness of the procedures shows a different outcome. With the reference procedure, several hundreds of samples can be analysed without any problem. Using the present surfactant-mediated clean-up procedure, a maximum of 150 samples could be processed on a single pre-column; after that, clogging of the pre-column started to occur. However, cleaning the pre-column is simple and inexpensive. With the present MLC procedure the robustness of the system was more restricted, because the back-pressure in the column gradually increased, which led to a slow deterioration of the performance starting after some 60 injections. Typically, after *ca.* 80 injections the upper layer of the pre-column and the screens of both columns had to be replaced. This problem could not be solved by lowering the columns temperature, by increasing the diameter of the inlet of the pre-column or by increasing the SDS concentration in the plasma sample. Despite the fact that other groups did not report significant back-pressure build-up [9–12], we did not succeed in solving this problem.

Considering the sample throughput on a 24-h time basis, with the reference procedure this is *ca.* 100 samples per day, the limiting factor being the time required for sample preparation. Using MLC, up to 150 samples per day can be determined, whereas with column-switching the throughput can be increased to 180 samples per day, with the chromatography being the limiting step.

CONCLUSIONS

The present study demonstrates that surfactant-mediated sample clean-up with a column-switching unit and, to a lesser extent, MLC, are good alternatives for the conventional determination of teniposide in plasma, which involves sample pretreatment based on liquid–liquid extraction. A main advantage of MLC is that it allows very simple manual pretreatment of plasma samples prior to liquid chromatogrpahy. Inherent drawbacks of MLC, which prevent its general applicability, are that no sample enrichment or clean-up can be carried out, which results in a moderate detection limit and selectivity, respectively. Furthermore, clogging of the column seems to further restrict the use of MLC in routine analysis.

Surfactant-mediated sample clean-up can easily be combined with sample enrichment. A major advantage of the column-switching procedure is that sample treatment can be fully automated, which is in marked contrast with the reference procedure, where a skilled technician is occupied almost full-time with sample preparation.

Despite the simplicity of the surfactant-based techniques, their analytical performance is comparable with that of the reference procedure.

Thus surfactant-mediated sample clean-up can be considered to be a simple and highly competitive technique for RP-HPLC determinations of drugs in plasma, which involve sample pretreatment based on liquid–liquid extraction.

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