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Note

Comparison of flow-injection analysis with high-performance liquid chromatography for the determination of etoposide in plasma

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Flow-injection analysis (FIA) and high-performance liquid chromatography (HPLC) show great similarity in instrumentation and in operational parameters. The main differences are basically caused by the fact that in FIA a reactor and in HPLC a separation column is used. The applications of flow-injection methods for analysis in complex matrices, which are for instance usually necessary in the clinical chemistry, are increasing steadily [1–4]. In the bioanalysis of drugs and/or their metabolites in biological fluids chromatographic methods are usually applied, whereas only a few flow-injection methods have been reported [5,6].

The present study compares a flow-injection method with an HPLC method for the determination of the antineoplastic agent etoposide in plasma. The flow-injection method is based on on-line electrochemical derivatization followed by spectrophotometric detection [6]. The applied HPLC method is a modification of an assay described by Holthuis et al. [7]. Spiked plasma samples are analysed by both methods. The results are evaluated statistically, providing specific estimates of the type and magnitude of errors [8,9]. The significance of the errors is tested at a confidence level of 95%. Accuracy, precision, selectivity and limit of determination are evaluated as the features that characterize the performance of the methods.

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EXPERIMENTAL

Chemicals and solutions

Etoposide (VP 16-213) and teniposide (VM 26) were kindly supplied by Bristol Myers (Weesp, The Netherlands). 1,2-Dichloroethane was freshly distilled before use. Other solvents and chemicals were of analytical grade and were used without further purification. All aqueous solutions were prepared with Millipore-Q water. Stock solutions (1.0 mg/ml) of etoposide and teniposide were prepared in methanol and diluted to 0.1 mg/ml with methanol. These methanolic solutions were stored at 4 °C and used to spike drug-free plasma.

Spiking, pretreatment and calibration

Blank plasma was obtained from healthy volunteers and spiked with etoposide. A duplicate set of spiked plasma samples (4.0 ml each) was prepared in the range 1–25 μg/ml in steps of 1 μg/ml. For this purpose appropriate volumes of the methanolic etoposide solutions (1.0 or 0.1 mg/ml) were transferred into a 10-ml polypropylene tube. The methanol was evaporated under nitrogen at room temperature, and 4.0 ml of blank plasma were added. Tubes were vortexed (1 min) to dissolve etoposide in the plasma. Each sample was divided between two polypropylene tubes. One portion was used in the flow-injection method and the other in the HPLC assay. These spiked plasma samples were frozen and stored at −18 °C.

Before the analysis plasma samples were extracted with dichloroethane. The organic phase was evaporated to dryness (nitrogen, room temperature) and the residue was dissolved in carrier (FIA) or mobile phase (HPLC). This pretreatment procedure was carried out as described earlier [6]. To the samples to be analysed by HPLC an internal standard (teniposide) was added before the extraction. An appropriate volume of a methanolic stock solution of teniposide (1.0 or 0.1 μg/ml) was transferred into a 4-ml polypropylene tube. The methanol was evaporated (nitrogen, room temperature), and 1.0 ml of spiked plasma was added. Furthermore, these HPLC samples were preconcentrated by dissolving the residue obtained after evaporation of the organic phase in only 150 μl of mobile phase instead of 500 μl as was used for FIA.

Calibration curves for FIA and for HPLC were measured in plasma. Calibration samples were freshly prepared. For this purpose appropriate volumes of the methanolic solutions of etoposide (FIA) or etoposide and teniposide as well (HPLC) were transferred into 4-ml polypropylene tubes. The methanol was evaporated and 1.0 ml of blank plasma was added. In order to obtain appropriate peak-height ratios the concentration of the internal standard has to be about the same as that of the analyte. Therefore internal standard was added at three different levels (2, 10 and 20 μg/ml) and three corresponding calibration curves in the ranges 1–5, 6–15 and 16–25 μg/ml, respectively, were measured.

Apparatus and conditions

The apparatus and conditions used for FIA were as described earlier [6]. The mean residence time was 1.0 min, allowing a sample throughput of 30 h^{-1}.

The chromatographic system consisted of a solvent-delivery system (Waters, Model 6000 A), an automatic injector [Waters Intelligent Sample Processor (WISP), Model 710 B], a guard column (5–10 μm, Lichrosorb RP-18, 20 mm × 3.9 mm I.D.), an analytical column (4 μm, Novapak phenyl, 75 mm × 3.9 mm I.D.) and a laboratory-made electrochemical detector cell [10] connected to a Metrohm 641 VA potentiostat. The oxidation current was measured at a potential of +500 mV vs. Ag/AgCl. The mobile phase was 10 m*M* phosphate buffer (pH 7)–methanol (45:55, w/w). The flow-rate was 1.0 ml/min. The chromatograms were recorded with a Kipp recorder (Model BD41), and peak-height ratios were measured. Amounts of 10–25 μl plasma extracts were injected. The retention times for etoposide and teniposide were 1.2 and 4.4 min, respectively. The chromatographic run could be performed within 6 min.

RESULTS AND DISCUSSION

Linearity and limit of detection

Calibration was performed in three concentration ranges (1–5, 6–15 and 16–25 μg/ml). Both FIA and HPLC showed good linearity in all ranges. The correlation coefficients were at least 0.999 for all curves. However, the intercepts of the FIA curves deviated significantly from zero for all ranges. The positive intercept in FIA can be explained by the blank response, which is fairly high owing to interfering plasma components. Therefore, the limit of determination (LOD) for the flow-injection method is governed by the blank response, whereas in HPLC the LOD is determined by the noise. The mean blank response ($n=5$) in FIA proved to be 0.78 mAU (S.D. = 0.19 mAU), corresponding to 0.68 μg/ml (S.D. = 0.17 μg/ml). The detector response that corresponds to the LOD is defined as y_{LOD}. The mean value of y_{LOD} can be calculated according $\bar{y}_{LOD} = \bar{x}_{bl} + k\text{S.D.}_{bl}$, where $\bar{x}_{bl}$ is the mean blank response, S.D._{bl} is the standard deviation of the blank response and k is the weighting factor, which equals the reciprocal relative standard deviation of the blank response [8]. The LOD in FIA proved to be 1.5 μg/ml. In HPLC the LOD is defined as the concentration corresponding to the response obtained at a signal-to-noise ratio of 3. The LOD was 0.15 μg/ml in the present HPLC procedure. The latter, however, can be lowered to 15 ng/ml by increasing the preconcentration factor and/or the injection volume.

Precision

The precision or reproducibility of a method refers to random (indeterminate) errors. The random error of a method can be investigated by evaluating the S.D. or the variance. In this study the precision of both methods was evaluated for concentrations ranging from 1 to 25 μg/ml. Each concentration was analysed in duplicate by both methods. The differences found between the duplicate determinations were defined as w. The variance (S.D.^2) in w was obtained from the relationship $\text{S.D.}^2 = w^2/2$. The mean variances for FIA and HPLC were calculated and proved to be 1.164 and 0.574 $(\mu g/ml)^2$, respectively, in the investigated concentration range of 1–25 μg/ml.

The *F*-test provides a simple method for comparing the precision of two meth-

ods. The experimental F value, F_{exp}, is calculated by dividing the larger variance by the smaller, and proved to be 2.03. The critical F value with 24 degrees of freedom in the numerator and in the denominator as well and at a confidence level of 95%, $F_{24;24;0.95}$, is 1.98. The precisions of the two methods differ significantly. The variance in HPLC is lower than in FIA, probably because of the use of an internal standard in HPLC.

Accuracy

In method comparison studies the accuracy can be investigated by evaluating the systematic errors, which include the constant and/or the proportional error. The t-test and the least-squares linear regression were used to estimate these errors. Since HPLC is the established method for the analysis of etoposide in plasma it is considered as the reference method, whereas FIA is the test method. Each spiked sample was analysed by both methods. The difference d is defined as the concentration found by FIA subtracted from the concentration found by HPLC. The mean difference was calculated, and the experimental Student t value, t_{exp}, was obtained according to $t_{exp} = (\bar{d}/\text{S.D.}_d)\sqrt{n}$. The mean difference, $\bar{d}$, is called the bias and provides an estimate of the systematic error of FIA with respect to HPLC. S.D._d, the standard deviation in d, provides an estimate of the random error in the bias. n is the number of samples analysed by both methods ($n=50$). Additionally the concentrations found by HPLC (x values) and FIA (y values) were statistically evaluated by linear regression (Fig. 1). The intercept (a) and slope (b) are estimates for the constant error and the proportional error, respectively. Both types of error are systematic errors. The scatter of the points around the least-squares line refers to random errors of FIA in relation to HPLC. This random error is estimated by the standard deviation in the residuals, S.D._r. The results are summarized in Table I and Fig. 1.

The t_{exp} was determined to be 3.46 ($n=50$). A systematic error of 0.96 was indicated by the t-test. The least-squares analysis showed that the flow-injection method dealt with both types of systematic error, a proportional error of 2.8% and a constant error of 0.60 μg/ml. The standard deviation terms, S.D._d and S.D._r agree with each other; they indicate a random error of 1.97 and 1.98 μg/ml, re-

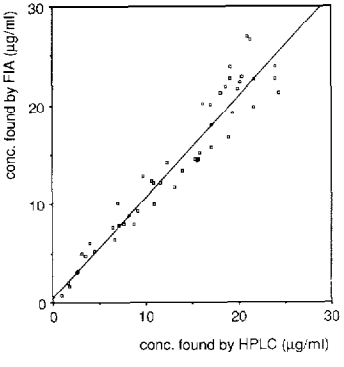

Fig. 1. Correlation between the concentration of etoposide in plasma samples as found by HPLC and by FIA.

TABLE I

PARAMETERS OBTAINED BY STATISTICAL ANALYSIS

Parameter	Value
t-test	
Bias (mean difference, $\bar{d}$)	0.96 μg/ml
S.D. in differences (S.D.$_d$)	1.97 μg/ml
Experimental t value (t_{exp})	3.96
Least squares	
Intercept (a)	0.60 μg/ml
Slope (b)	1.028
S.D. of residuals (S.D.$_r$)	1.98 μg/ml
Correlation coefficient (r)	0.9658

TABLE II

STATISTICAL EVALUATION

	Expression	Found	Degrees of freedom
Sum of squares (SS)			
Slope (SS_b)	$(b-1)^2\sum(x_i-x)^2$	1.97	1
Intercept (SS_a)	$n(y-x)^2$	46.1	1
Scatter (SS_s)	$\sum(y_i-y-b(x_i-x)^2)$	187	$n-2$
F-test			
Slope (F_s)	SS_b/SS_s	0.50	
Intercept (F_i)	SS_a/SS_s	11.8	

spectively. This means that the 95% confidence interval of a measured value, x, equals $x \pm 3.88$ μg/ml. The dimensionless correlation coefficient, r, usually the only parameter applied in method comparison studies, provides little information. It is only sensitive to one type of error, the random error, but it is not an estimate for the magnitude of this error.

Finally some statistical tests were performed to conclude whether the estimated errors are significant. The experimental t value, t_{exp}, was compared with the critical t value of 2.01 (49 degrees of freedom and 95% confidence level). Thus the FIA method shows a significant systematic error. To conclude whether FIA dealt with a significant proportional error or/and a significant constant error, two F tests were performed. Firstly the sum of squares of the slope (SS_b), of the intercept (SS_a) and of the scatter (SS_s) were calculated according the expressions summarized in Table II [8]. Then F_{slope} (F_s) and $F_{intercept}$ (F_i) can easy be calculated (Table II). Both have an F-distribution with 1 and $(n-2)$ degrees of freedom in the numerator and in the denominator, respectively. The critical F value at the 95% confidence level ($F_{1;48;0.95}$) is 4.05. By comparing this value with the F_s and F_i (Table II) it is shown that the slope does not deviate significantly from 1, whereas the intercept deviates significantly from 0, which points to a constant error in FIA with respect to HPLC. The blank response cannot account

for this constant error because standards as well as samples have been measured in plasma. Since different blank plasma batches were used it can be concluded that the blank plasma varies from batch to batch, leading to a variable blank response.

CONCLUSIONS

FIA of etoposide in plasma is a good alternative to HPLC analysis, when the plasma levels are at least 1.5 μg/ml. The rapid stabilization, the flexibility and the high speed of analysis makes FIA attractive for routine control analysis of patient plasma. Usually, the drug concentration in patient plasma is rather high and the use of FIA for drug monitoring may be not limited by its fairly high LOD value.

Studies have been started to improve the precision and the selectivity of FIA by applying multi-channel detection with a UV photodiode array detector. It enables the use of an internal standard based on spectral differences between analyte and internal standard. Furthermore, when the spectrum of blank plasma differs from the spectrum of the analyte it allows spectral separation between blank and analyte, resulting in enhanced selectivity.

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