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Simultaneous determination of cytosine arabinoside, daunorubicin and etoposide in human plasma

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ABSTRACT

A method for simultaneous bioanalysis of the three cytotoxic drugs cytosine arabinoside, daunorubicin and etoposide in human plasma was developed and validated. A HPLC method with ultra-violet and fluorescence detection, preceded by mixed-mode cation-exchange solid phase extraction sample preparation, was used for the quantification of the analytes. The assay was used for the simultaneous measurement of cytosine arabinoside, daunorubicin and etoposide with linearity in the ranges of 13–1500 ng/mL, 15–1000 ng/mL and 52.5–3500 ng/mL, respectively. The chromatographic run-time was 15.5 min. The overall precision (% relative standard deviation) was within 0.2–13.5% and the recovery ranged between 86.1% and 110.1% for the three drugs at all concentrations tested. Plasma samples were stable for at least two months when stored at −20 °C. The method was successfully applied to quantification of the three drugs in blood samples from patients undergoing induction treatment for acute myeloid leukaemia, thus demonstrating its suitability for clinical studies.

1. Introduction

Acute myeloid leukaemia (AML) represents a group of clonal hematopoietic stem cell disorders in which both failure to differentiate and over proliferation in the stem cell compartment result in accumulation of non-functional cells [1]. AML may be treated with the complex ADE-induction therapy comprising the antimetabolite cytosine arabinoside (Ara-C, Fig. 1) for 10 days (b.i.d. on days 1–10), the anthracycline daunorubicin (Dnr, Fig. 1) for three days (q.d. on days 1, 3 and 5), and the podophyllotoxin-derivative etoposide (Eto, Fig. 1) for five days (q.d. on days 1–5) with the goal to remove all hematopoietic elements from the bone marrow [1,2]. Treatment with these anticancer drugs are primarily individualised to patient body surface area, and result in a high interpatient drug concentration variability in both plasma and leukemic cells [3–6]. This prompts for population based pharmacokinetic–pharmacodynamic analysis, where relevant co-variates such as liver and kidney function, age, sex, concomitant medication, etc. can be tested for influence on the PK–PD data. It may be possible to individualise future doses more accurately, and potentially eliminate many of the difficulties associated with the solely BSA-based dose adjustments, if statistically significant co-variates are modelled in concert with PK–PD data [6,7]. The ADE treatment has recently been introduced as a standard induction treatment with the AML15 and 17 trials [webA, webB], and no simultaneous bioanalysis of the three drugs in human plasma has yet been described. Such an assay would facilitate data generation for population PK–PD analysis. A citation of all published bioanalytical quantification methods for these three compounds is not possible, but previously reported methods for Ara-C include HPLC-UV [8,9], HPLC–MS/MS [10] and RIA [11]. Methods for Dnr mainly employ HPLC-FLD [8,12,13] and HPLC–MS/MS [14] but also CE-LIF methods have been reported [15,16]. Methods for Eto include HPLC-FLD [17], HPLC–MS [18] and HPLC-ED [19]. A number of review papers with more exhaustive summaries of the different methods have been published; e.g. for Ara-C [20], for Dnr [21] and for Eto [22]. This paper introduces a bioanalytical HPLC-UV/FLD method with SPE extraction, capable of simultaneous sample preparation, detection and quantification of all three drugs. The method was validated and its clinical usefulness was shown in the bioanalysis of blood samples from patients in ADE-induction treatment for AML.

Abbreviations: ADE, cytosine arabinoside + daunorubicin + etoposide treatment; AML, acute myeloid leukaemia; Ara-C, cytosine arabinoside; BSA, body surface area; CE, capillary electrophoresis; Dnr, daunorubicin; ED, electrochemical detection; Eto, etoposide; FLD, fluorescence detection; HPLC, high pressure liquid chromatography; LIF, laser induced fluorescence; MS, mass spectrometry; PK–PD, pharmacokinetic–pharmacodynamic; RSD, relative standard deviation; SD, standard deviation; SPE, solid phase extraction; THU, tetrahydrouridine; t_R, retention time; UV, ultra-violet.

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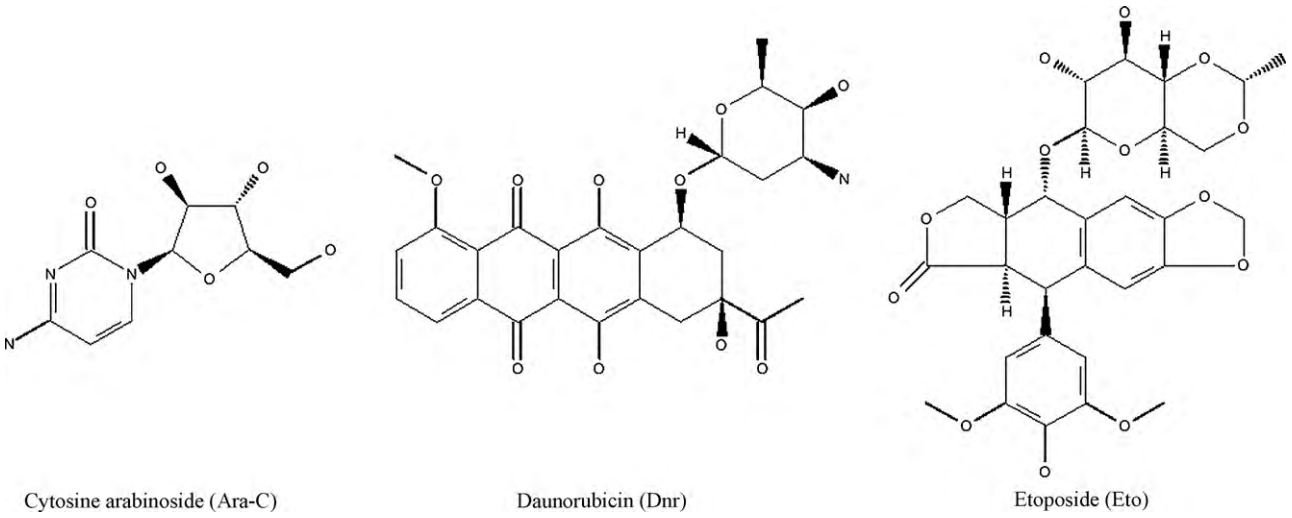

Fig. 1. Structure formulas of Ara-C, Dnr and Eto.

2. Experimental

2.1. Drugs and reagents

Ara-C, Dnr and Eto were all purchased from Sigma–Aldrich, Denmark. Tetrahydrouridine (THU, deaminase inhibitor) was purchased from VWR, Denmark. Waters Oasis® MCX 3cc, 30 μm (60 mg) extraction cartridges were purchased from Waters, MA, USA. All other chemicals and solvents were of analytical grade.

2.2. Instrumentation

The HPLC equipment was from the Agilent 1100 series, except the fluorescence detector which was from the Agilent 1200 series. The equipment consisted of a G1365B MWD UV detector, a G1312A FLD fluorescence detector with a standard 8 μL FLD cell, a G1316A COLCOM column oven, a G1313A ALS autosampler, a G1376A Cap-Pump pump and a G1379A Degasser. The software used for analysis of the signals was Chemstation for LC systems [rev. B.03.02-SR2] from Agilent technologies, Denmark. The column was an Acclaim Polar Advantage II (4.6 × 150 mm, 3 μm) C_{18} from Dionex, Denmark.

2.3. Sample preparation

Solutions (1 g/L) of Ara-C in H_2O, Eto and Dnr in methanol, were further diluted to 100 mg/L with 5 mM ammoniumformiate pH 4.6, and kept at −20 °C. Calibration curves were made from further dilutions of these stock solutions. Spiked plasma samples were prepared with plasma from a healthy human donor. The final spiked plasma sample consisted of 5 μL THU solution (1 mg/mL), a variable volume of analyte stock solution, and then plasma to a final volume of 600 μL. The volume of analyte stock solution did not exceed 54 μL (i.e. 9% of total sample volume), and the final percentage of methanol in the samples did not exceed 0.45%. THU was added to the samples to prevent the cytidine deaminase conversion of Ara-C to the inactive metabolite Ara-U [23]. The analytes were separated from interfering substances in the plasma matrix by solid phase extraction using Waters Oasis MCX cartridges. The cartridges were conditioned with 2 mL methanol and 2 mL 0.05 M HCl. Plasma samples were diluted 1:1 with 0.05 M HCl and 500 μL sample was placed on the column. The column was washed with 1 mL 0.05 M HCl and 1 mL 30% methanol. The analytes were eluted with 1 mL NH_4OH:MeOH:MeCN (10:95:95) twice. The combined eluates were evaporated under nitrogen at 60 °C, and the remanence was redissolved in 250 μL of mobile phase A before injection into the HPLC.

2.4. Patient samples

Factors that could influence the degradation of analytes in the patient samples were validated with blood samples from a single patient, and with spiked whole blood samples from a healthy volunteer. The effects of storage time and temperature before centrifugation as well as the effect of THU addition were tested. For the storage time experiment, spiked whole blood stored at 4 °C was analysed at four time points: 0 h, after 0.5 h, after 2 h, after 4 h and after 12 h. Blood samples were taken from patients in induction treatment for AML: Ara-C (100 mg/m^2), Dnr (50 mg/m^2) and Eto (100 mg/m^2). Following treatment, blood samples were collected into heparinised tubes containing THU (0.1 mg THU/mL blood). Blank samples were collected immediately before the first treatment to ensure that there were no interfering peaks in the chromatograms. All samples were stored at 4 °C until further treatment. Plasma was separated by centrifugation at 2500 × *g* for 15 min within 4 h after the sample was taken. An aliquot was analysed immediately and the remaining plasma was kept at −20 °C.

2.5. Ethics

The project was approved by the national ethics committee (Journal no. H-A-2008-129).

2.6. HPLC conditions

A gradient elution programme was developed with the mobile phases A (3 g/L KH_2PO_4, adjusted to pH 2.0 with concentrated phosphoric acid) and B (acetonitrile). The gradient was: $t_{0\,min} - t_{1.5\,min}$: 0%B; $t_{1.5\,min} - t_{3\,min}$: linear increase to 57%B, which was maintained until $t_{6.5\,min}$, and then another linear increase from $t_{6.5\,min} - t_{7\,min}$ to 62%B, which was maintained until $t_{11\,min}$; from $t_{11\,min} - t_{11.5}$ the %B was reduced to 0% and the system was equilibrated in this way for an additional 4 min to be ready for the next run. The flow rate was 1 mL/min, the column temperature 30 °C and 50 μL sample was injected into the system. The UV detection was set to 280 nm. The FLD excitation and emission wavelengths were 230 nm and 328 nm, respectively, for the first 10 min (for Eto detection) and

Table 1
Data from the validation procedure.

Analyte	Spiked concentration (ng/mL)		Precision inter-run (% RSD; $n=5$)	Precision intra-run (% RSD; $n=4$)	Accuracy (%; $n=5$)	Recovery (mean, %; $n=5$)	12 h post-preparative stability ($t_{12}/t_0 \times 100\%$; $n=3$)
Ara-C	ULoQ	1500	2.1	0.6	97.8	110.1	106.7
	MED	450	1.8	0.4	100.7	106.5	105.3
	LOW	30	8.7	6.3	99.2	102.3	97.0
	LLoQ	13	13.5	7.2	98.2	94.0	104.2
Dnr	ULoQ	1000	4.2	0.5	115.0	86.1	104.3
	MED	300	5.3	0.4	94.3	108.4	106.0
	LOW	20	7.2	2.1	101.5	90.3	106.0
	LLoQ	15	10.4	5.1	103.2	99.5	107.1
Eto	ULoQ	3500	4.8	0.2	102.3	97.0	99.8
	MED	1050	2.6	2.5	101.2	103.2	101.0
	LOW	70	4.2	9.5	99.5	96.9	99.9
	LLoQ	52.5	12.0	7.2	102.9	99.4	98.2

then switched to 490 nm and 555 nm, respectively, (for Dnr detection) for the remaining 5.5 min.

2.7. Validation: precision, accuracy, selectivity, recovery stability and dilution

The described tests for validation were all carried out based on the "Guidance for Industry – Bioanalytical Method Validation" document by FDA [webC]. The lower limit of quantification (LLoQ) was defined as the analyte response giving at least five times the response compared to blank response. For the calibration curves, seven calibrator points with four replicate samples were used for each analyte. The inter-run precision and recovery of the method was analysed by performing SPE and HPLC on five separate samples of spiked plasma at four different concentrations: LLoQ, low, intermediate and upper limit of quantification (ULoQ). Intra-run precision was assessed by injecting the same sample four times in sequence. The precision was expressed as the relative standard deviation (%RSD) in per cent of the detector response from the five samples. The limits of acceptance were a %RSD below 20% at LLoQ, and below 15% for all other concentrations. The recovery was expressed as the percentage of the results from extracted plasma samples to the results from aqueous calibration standards with the same concentrations, diluted in mobile phase A and not subjected to SPE. The accuracy was determined by replicate analysis of five samples at the four concentrations, and was calculated as the percentage of the measured concentration to the known concentration. Selectivity was tested by comparing spiked plasma samples with blank plasma samples from healthy volunteers, as well as by comparing patient samples before and after treatment. In total, plasma from six different subjects was tested in the selectivity tests, to ensure the robustness of the method. To test the post-preparative stability, the processed samples were left standing in the autosampler for 12 h at ambient temperature, and then analysed again. In order to detect any loss of the three analytes during long time storage, plasma from the patients was kept at −20 °C for two months and then analysed again. Stock solution stability was tested by comparing solutions kept at −20 °C for two months with freshly prepared stock solution. Freeze–thaw stability was assessed with spiked plasma (at ULoQ and LLoQ) from a healthy volunteer stored at −20 °C for 24 h and then thawed at room temperature. The samples were refrozen and thawed three times in total, and the detector responses from injections were compared to freshly spiked plasma samples. Dilution procedures were validated by spiking blank plasma with stock solutions to a concentration five times the ULoQ, and then diluting this 10 times with phosphate buffered saline, pH 7.4. The diluted plasma was extracted with SPE and analysed. The limits of acceptance for the calculated concentrations relative to the nominal concentrations were 85–115%.

3. Results and discussion

A method for simultaneous bioanalysis of the three cytotoxic drugs Ara-C, Dnr and Eto in human plasma was developed and validated, enabling future pharmacokinetic analysis based on blood samples from patients with acute myeloid leukaemia.

3.1. Development of method: extraction

Initially simple protein precipitation sample clean-up methods with cold perchloric acid as well as with acetonitrile were inves-

Table 2
Data from patient samples. Infusion times were Ara-C: 5 min, Dnr and Eto: 1 h.

Patient	Analyte	Amount infused (mg)	Time after infusion (min or h)	Measured concentration (ng/mL)	Two-month stability at −20 °C (conc. $t_0/t_{2\,\text{months}}$, %)
P1 (59 years, female, BSA 1.5)	Ara-C	150	10 min	1561	100.3
	Ara-C	150	15 min	434	*
	Dnr	75	40 min	35	106.6
	Dnr	75	105 min	25	*
	Eto	150	24 h	263	*
	Eto	150	35.5 h	125	107.5
P2 (47 years, male, BSA 1.9)	Ara-C	190	20 min	270	95.4
	Ara-C	190	35 min	134	*
	Dnr	95	35 min	174	100.0
	Dnr	95	145 min	27	*
	Eto	190	100 min	7105	*
	Eto	190	9.5 h	2271	95.7

* Not analysed.

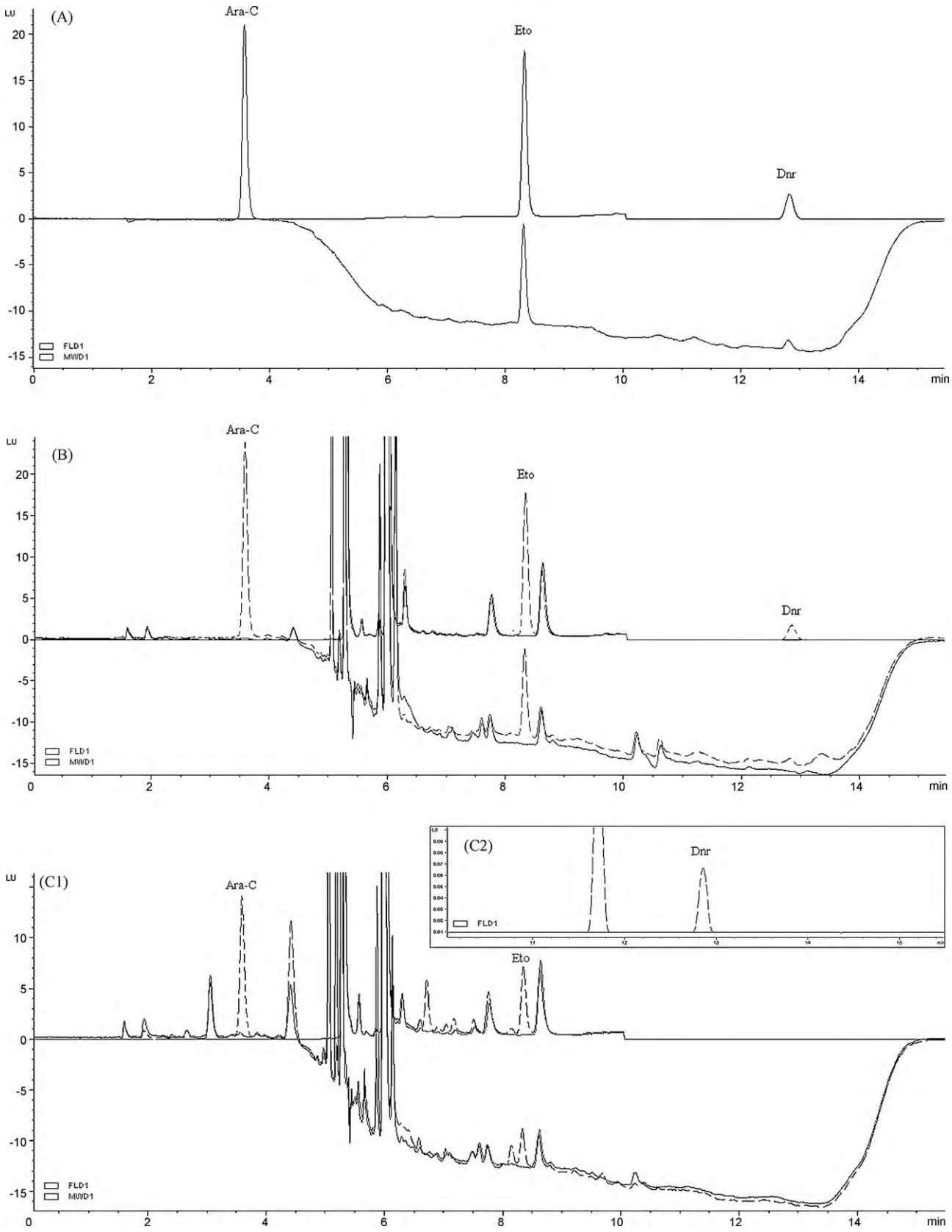

Fig. 2. Chromatograms. (A) An injection of pure Ara-C, Eto and Dnr diluted in 3 g/L KH_2PO_4, pH2. (B) Injections of blank plasma (full line) and spiked plasma (dotted line) from a healthy volunteer. The concentrations in the spiked plasma were: Ara-C: 1500 ng/mL, Eto: 3500 ng/mL, Dnr: 1000 ng/mL. (C1) Injections of a blank blood sample before treatment (full line) and a blood sample after treatment with Ara-C and Eto from a patient with AML (dotted line, measured concentrations were 505 ng/mL for Ara-C and 1742 ng/mL for Eto). The insert (C2) shows the blank blood sample (full line) and another blood sample from the same patient after treatment with Dnr (dotted line, measured concentration was 46 ng/mL). The retention times were 3.6 min for Ara-C, 8.4 min for Eto and 12.8 min for Dnr.

Table 3
Weighted ($1/x^2$) calibration curves for the three drugs in plasma.

Analyte	Regression line	R^2	SD_{slope}	$SD_{Y\text{-intercept}}$
Ara-C	$Y = 0.146x + 0.541$	0.989	0.003	0.088
Dnr	$Y = 0.066x - 0.384$	0.966	0.002	0.077
Eto	$Y = 0.069x - 2.282$	0.987	0.002	0.170

SD: standard deviation.

tigated. This resulted in unacceptable, low recoveries for Dnr and Eto (data not shown). Since Dnr and Eto are protein bound (60% and 94%, respectively) to a higher degree than Ara-C (13%) [24], it was speculated that Dnr and Eto co-precipitated with plasma proteins. However, addition of sodium dodecyl sulphate in order to increase the solubilisation of plasma proteins and thus release protein-bound Dnr and Eto [25], did not increase the recoveries. Therefore, a solid phase extraction was employed. Due to the different physicochemical properties of the three analytes, a strong cation-exchange mixed-mode polymeric sorbent (Oasis MCX) was chosen. This SPE column was capable of both ion-exchange and reversed-phase interactions. Plasma was diluted with 0.05 M HCl and the cartridges were preconditioned with 0.05 M HCl in order for the $-NH_2$ group in both Ara-C ($pK_a \sim 4.2$) and Dnr ($pK_a \sim 8.4$) to be completely ionised, while the –OH group of Eto was left unionised ($pK_a \sim 9.8$). A relatively low concentration of methanol (30%) was used during the wash phase to avoid unnecessary loss of the more lipophilic and unionised Eto. The mixture of acetonitrile, methanol and ammonia proved to be adequate to release Ara-C and Dnr from the ion-exchange interaction and to release Eto from the reversed-phase interactions, resulting in recoveries above 86.1% (Table 1). During development of the method a smaller peak eluting just before the Eto peak could be seen in the chromatograms, and, in a separate experiment, this peak was reduced when the alkaline conditions of the eluent were reduced. It was suspected that Eto was turned into the corresponding cis-lactone with the alkaline conditions of the elution in the SPE [26]. To prevent this transformation from trans-Eto to cis-Eto as much as possible, the evaporation temperature was set at a relatively high 60 °C in order to minimise the time spent on this step, and the amount of NH_4OH in the eluent was as low as possible without reducing the recovery of Ara-C and Dnr. A separate experiment was carried out, in which a standard mixture of the three analytes were injected directly into the HPLC, or evaporated at either 50 °C or 60 °C, then redissolved in mobile phase A and injected. The peak areas for the three injections were compared, and no difference was seen (data not shown). This demonstrated that no analyte was lost despite the relatively high evaporation temperature.

3.2. Development of method: HPLC

The retention times for Ara-C, Eto and Dnr in plasma in the final method were 3.6 min, 8.4 min and 12.8 min, respectively. Chromatograms of the pure standards diluted in mobile phase A, blank and spiked healthy human plasma, and plasma from two patient samples are shown in Fig. 2. Different mobile phase systems were evaluated during method development and optimisation. With the same gradient system, a change of mobile phase B from acetonitrile to methanol changed the order of elution of Eto ($t_{R,MeOH} = 7.3$ min) and Dnr ($t_{R,MeOH} = 6.7$ min), and decreased the final run-time to 11 min. However, with methanol as the organic mobile phase B, the Eto peak was seen at a time where the gradient caused a steep baseline drift in the chromatograms, which would make the quantification of Eto less reliable. Both peak heights and peak areas were evaluated for quantification, and since no difference was found between the two methods, peak areas were chosen for all quantifications. The choice of column was based on its ability to be used with 100% aqueous mobile phase, in order to increase the Ara-C retention on the column as much as possible. With even as little as 5–10% organic content in the mobile phase, the Ara-C eluted too close to the solvent front, and also tended to co-elute with plasma matrix compounds.

3.3. Development of method: patient samples

HPLC analysis showed no difference in the amount of the three drugs when the whole blood samples were stored at 4 °C for 4 h before separation of plasma by centrifugation, compared to samples stored at 4 °C for 0 h, 0.5 h and 2 h before centrifugation (data not shown). Storage for 12 h at 4 °C increased the degree of haemolysis to such an extent, that the separated plasma was visually different from the plasma obtained with a shorter storage time, in concert with previous reports [27]. It has been reported that anthracyclines are unstable in whole blood, because they enter the cells and become a substrate for aldo–keto reductase enzymes [28]. The maximum storage/transport time was set to 4 h at 4 °C, in order to avoid uncertainties associated with haemolysis and possible enzymatic degradation. As could be expected, addition of THU to the blood sample vials completely removed the degradation of Ara-C by cytidine deaminase [23]. The successful quantification of the three drugs in patient blood samples (Table 2) shows that the method can be used for clinical studies.

3.4. Limits of quantification and calibration curves

The lower limit of quantification was 13 ng/mL for Ara-C in plasma. In comparison to other HPLC-UV methods, Burk et al. [9] report a LLoQ of 10 ng/mL for Ara-C in human plasma. For Eto the LLoQ in plasma was 52.5 ng/mL. With SPE-HPLC-FLD, Manouilov et al. [17] reported a LLoQ of 500 ng/mL, and a limit of detection of 200 ng/mL for etoposide in human serum. For Dnr the LLoQ in plasma was 15 ng/mL. de Jong et al. [12] have reported a HPLC-FLD analysis method for Dnr with linearity in the range 1–250 nM (i.e. 0.53–132.5 ng/mL) in plasma, Maudens et al. [13] have recently reported a LLoQ of 2.5 ng/mL, while Hulhoven and Desager [29] in an older study reported a detection limit of 10 ng/mL in plasma with HPLC-FLD. The method reported with this paper has thus an improved LLoQ for Eto and a similar LLoQ for Ara-C, but a higher LLoQ for Dnr. However, the limits are sufficiently low to be clinically relevant, and must be seen as a compromise needed for analysing all three drugs at the same time.

A weighting of the data for the calibration curves was considered by using the same method as Maudens et al. [13], where unweighted, $1/x$ weighted and $1/x^2$ weighted linear regression analysis was compared by sum of residual error and by scatter plots and residues plots. For all three analytes a weighting with $1/x^2$ gave the best linear regression model to adequately describe the curves. The weighted calibration curves and corresponding R^2 values and standard deviations are listed in Table 3.

3.5. Validation: precision, accuracy, selectivity, recovery, stability and dilution

The range of %RSD for the measurements at LLoQ, low, intermediate and ULoQ concentrations were within the limits of acceptance, as stated in Section 2.7, for both intra-run and inter-run precision (Table 1). The mean recovery was 99.5% (range 86.1–110.1%) for Ara-C, Dnr and Eto at all four concentrations. The accuracy assessment was acceptable as well, but it should be noted, that the limit of 15% deviation only just held true at the ULoQ (Table 1). There were no interfering peaks in any of the four blank plasmas from four different volunteer subjects. This fact together with the chromatograms of the samples from the two

patients demonstrated an adequate robustness of the selectivity of the method (Fig. 2). Patients in treatment for AML receive a multitude of different drugs besides the cytotoxic drugs, e.g. antiemetics, antibiotics, anxiolytics and diuretics. It would be very difficult to test for interferences for all concomitant medication, as well as metabolites, and this is a weakness of the method. It is, however, a good indication of selectivity, that no peaks were observed from blood samples collected from patients during treatment at time points where none of the analytes were expected to be present in plasma, and where peaks originating from concomitant medication and metabolites could be expected to be present. The post-preparative analysis showed that the samples were stable for up to 12 h when left standing at ambient temperature in the HPLC autosampler (Table 1). The storage stability showed that no amount of analyte was lost when plasma was stored at −20 °C for two months (Table 2). The stock solution was stable for at least two months at −20 °C, with $t_{2\,\mathrm{months}}$ peak areas relative to t_0 peak areas of 99.6%, 96.4% and 106.1% for Ara-C, Dnr and Eto, respectively. The freeze–thaw stabilities of the spiked samples were within limits, with a peak area relative to freshly spiked plasma between 89.8% and 113.1% for the three analytes at the two concentrations tested (ULoQ and LLoQ). Dilution was validated since especially Eto concentrations can be above ULoQ shortly after infusion (Table 2, patient 2). The mean concentrations measured after dilution relative to the nominal concentrations in percent were for Ara-C: 93.6%, for Dnr: 109.7% and for Eto 91.2%. The method was considered reliable and reproducible since all the measurements were consistent and within the limits of acceptance regardless of the concentration of analytes (Table 1), and since spiked plasma samples will be used as calibration standards in each future bioanalysis.

3.6. Conclusion

In conclusion, a SPE-HPLC-UV/FLD method was developed and validated for the simultaneous analysis of the three anticancer drugs Ara-C, Dnr and Eto. The clinical applicability of the method was demonstrated with blood samples from AML patients. This method is clinically relevant because of the complex treatment schedule used in the induction treatment of AML patients, where, depending on the day and time of treatment, Ara-C, Dnr and Eto are administered either as only Ara-C or Ara-C together with Eto, or Ara-C and Eto together with Dnr. The order, in which the infusions are given if all three drugs are to be infused, might also change from day to day, or from patient to patient. The developed method will therefore ensure that no information on plasma concentrations is lost regardless of the time-point for blood sampling, and at the same time, that no unnecessary analysis will be performed due to a wrong choice between corresponding single-analyte methods. The method will be used in a clinical trial for population based pharmacokinetics of the induction treatment of AML, with the aim to provide a more refined individualised treatment.

Conflict of interest statement

The authors declare no conflict of interest.

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