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Sensitive high-performance liquid chromatographic method for the determination of N^4-hexadecyl- and N^4-octadecyl-1-β-D-arabinofuranosylcytosine in plasma and erythrocytes

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Abstract

N^4-Hexadecyl- and N^4-octadecyl-1-β-D-arabinofuranosylcytosine (NHAC, NOAC) are two new cytostatic derivatives of cytosine arabinoside (ara-C) with improved cytostatic activity and stability against deamination. A high-performance liquid chromatography (HPLC) method was developed for the specific determination of NHAC and NOAC in plasma and erythrocytes, after solid-phase extraction using UV detection at 275 nm. Because of the strong binding of the drugs to proteins and membranes, the samples have to be pretreated with urea (plasma) or butanol and ultrasonication (erythrocytes). The calibration curves are linear for both drugs ($r > 0.999$) in the concentration ranges 20–2100 μg/l for plasma and 40–4200 μg/l for erythrocytes, respectively. The within-day and between-day precision studies showed a good reproducibility, with coefficients of variation below 8.5%. The recoveries of the lipophilic ara-C derivatives are greater than 66%. The method described can be applied to pharmacokinetic studies with NHAC and NOAC.

1. Introduction

1-β-D-Arabinofuranosylcytosine (ara-C) is an effective chemotherapeutic agent for the treatment of acute myelogenous leukemia [1–3]. Because of its rapid deamination to the biologically inactive metabolite 1-β-D-arabinofuranosyluracil (ara-U) [4,5], the drug has to be administered intravenously, e.g. as a continuous infusion over 5 days or as a high-dose regimen [6,7]. To increase the stability of the active drug against enzymatic deamination, several derivatives of ara-C have been developed by modifying mainly two positions in its molecular structure. At the carbon atom in position 5 of the arabinofuranoside acyl chains [8], steroids [9] or phospholipids [10] were attached. However, significantly increased stability was not achieved, which is not unexpected because the amino group is unmodified and still exposed to deamination. In other approaches different acyl [11,12] and alkyl [13] chains were linked to the amino group of cytosine, but protection was only partially achieved with the acyl derivatives [11]. Derivatives containing an alkyl chain of 16 or 18 carbon atoms (N^4-hexadecyl- and N^4-octadecyl-

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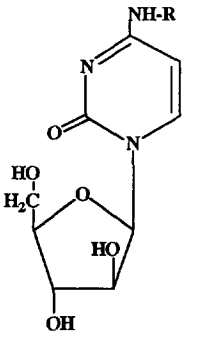

R =	Name	Abbreviation
H	1-ß-D-Arabinofuranosylcytosine	ara-C
$C_{16}H_{33}$	N4-hexadecyl-1-ß-D-arabinofuranosylcytosine	NHAC
$C_{18}H_{37}$	N4-octadecyl-1-ß-D-arabinofuranosylcytosine	NOAC

Fig. 1. Structural formulae of ara-C, NHAC and NOAC.

1-β-D-arabinofuranosylcytosine, NHAC and NOAC) resulted in the most resistant compounds (Fig. 1). These long-chain alkyl derivatives are highly lipophilic and, therefore, must be incorporated into small unilamellar liposomes to allow parenteral application.

These two new drugs exerted a significantly increased cytostatic activity in the L1210 mouse tumor model, compared with ara-C. Experiments performed to determine the curative dose after inoculation of L1210 leukemia in mice revealed that the dosages of NHAC or NOAC needed are significantly lower than that of ara-C [14]. With in vitro experiments it was demonstrated that the cytostatic effect of NHAC and NOAC is predominantly independent of the formation of ara-C and phosphorylated metabolites [15]. The exact mechanism of action is currently under investigation. Because of their resistance to cytidine deaminase, NHAC and NOAC also showed a curative cytostatic effect in the L1210 mouse leukemia model when administered orally [16]. In vitro studies describing the interactions of NHAC with blood components showed that the drug binds rapidly and with high efficiency to erythrocytes, leukocytes, and plasma proteins [17].

Further studies are ongoing to determine the pharmacokinetic properties of NHAC and NOAC in mice and humans. A phase I dose escalation study of NOAC has been initiated in our hospital along with pharmacokinetic monitoring of the plasma and erythrocyte concentration of NOAC using the analytical methods presented in this paper.

2. Experimental

2.1. Chemicals

Laboratory-grade ammonium formate, butanol, and urea and HPLC-grade dichloromethane were obtained from Fluka Chemie (Buchs, Switzerland); HPLC-grade methanol was obtained from Merck ABS (Dietikon, Switzerland); NHAC and NOAC were synthesized by H. Schott (Tübingen, Germany) [13,14]; tetrahydrouridine (THU) was a generous gift of the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Rockville, MD, USA. The THU solution was prepared by dissolving 1 mg/ml in water.

2.2. HPLC

The HPLC system consisted of a 9010 pump, a 9100 autosampler and a 9050 UV–Vis detector (Varian, Sunnyvale, CA, USA) and a column heater Croco-cil (Cluzean-info-labo, Saint-Foy-la-Grande, France). The detector was set at 275 nm, and the autosampler was equipped with a 92-μl loop. Two identical columns (Nucleosil C_{18}, 5 μm particle size, 120 Å pore size, 250 × 4 mm I.D.) were connected in series, and protected with a guard column (11 × 4 mm) packed with the same material. The first column was kept at room temperature, the second at 45°C. The mobile phase consisted of a mixture of methanol (90%) and 0.16 *M* ammonium formate buffer (10%) (pH 2.7). To minimize the background noise, the solvent mixture was prefiltered with 0.22-μm modified polyvinylidene difluoride filters (Millipore, Bedford, MA, USA). The flow-rate of the mobile phase was set to 0.9 ml/min.

2.3. Sample preparation

Plasma

To minimize deamination of NHAC or NOAC, 250 μl of THU solution were added to heparin-coated Vacutainer tubes (Becton Dickinson, Meylan Cedex, France) before acquisition of 10 ml venous blood from patients. Plasma and blood cells were separated by centrifugation (2800 *g*, 10 min). To 1 ml of plasma (standards, controls, or samples) 100 μl of internal standard (6.25 μg/ml, NHAC for the analysis of NOAC and vice versa) and 1 ml of a 10 *M* solution of urea were incubated overnight at room temperature on a horizontal shaker at 100 rpm. Solid-phase extraction (SPE) was performed using Bond Elut, C_{18} cartridges (Varian). After pre-washing the cartridges with 10 ml distilled water, 10 ml methanol, and 10 ml distilled water, the entire volume of the incubated sample was applied to the cartridge, followed by 1 ml distilled water and 0.5 ml 33% methanol. NHAC and NOAC were eluted with 3 ml methanol. The methanol was dried by evaporation (Rotavapor, Büchi, Flawil, Switzerland) and the residue dissolved in 150 μl mobile phase.

Erythrocytes

To 0.5 ml of erythrocytes suspended in 0.5 ml of THU solution (standards, controls, or samples) 100 μl of internal standard (6.25 μg/ml, NHAC for the analysis of NOAC and vice versa), 1 ml phosphate-buffered saline (pH 7.4), and 1 ml butanol were added, and the mixture sonicated for 2 h (Branson, 90W, Bransonic, Shelton, CT, USA), followed by centrifugation at 4800 *g* for 10 min. With the supernatant SPE was performed as described for plasma. To the solid residues 3 ml methanol were added and the mixture sonicated for 30 min. The samples were then centrifuged at 4800 *g* for 10 min. The methanol solution was immediately pipetted into a clean vial. This procedure was repeated once. All methanolic extracts of NHAC or NOAC were combined and the solvent evaporated. For HPLC analysis the residues were dissolved in 200 μl mobile phase.

2.4. Linearity

Corresponding known amounts of a NHAC or NOAC solution in methanol were added to human plasma or erythrocyte suspension for the preparation of 6 standards whose concentrations ranged from 20 to 2080 μg/l in plasma and from 40 to 4200 μg/l in erythrocytes, respectively. These standard samples were extracted as described above and the standard curves plotted as the peak area ratio of the respective compound to the internal standard versus the concentration. To assess linearity, the line of best fit was determined by least square regression.

2.5. Precision and accuracy

To determine the analytical precision, two plasma samples were prepared by addition of either 498 and 1478 μg/l NHAC or NOAC. For the determination of the precision in the erythrocyte suspension, two samples were spiked with either NHAC or NOAC to obtain concentrations

of 996 and 2956 μg/l, respectively. On four different days a calibration curve for each type of analysis was recorded, and the two samples were analysed once. For the determination of the within-day precision, the samples were run four times on the same day. To obtain the within-day and between-day coefficients of variation, mean and standard deviations were calculated for each series of analyses.

The accuracy of the method was assessed by expressing the mean of the assayed concentration for the precision samples as percentage of the weighed-in concentration.

2.6. Recovery

For determination of the recovery, 2.1 μg of NHAC or 2.5 μg of NOAC were added either to 1 ml of plasma or erythrocyte suspension ("sample") or to 150 μl or 200 μl of mobile phase ("standard"), respectively. The samples were extracted as described above. These analyses were performed in triplicate, and the average peak area of each compound of the sample was compared with the corresponding peak area of the standard.

2.7. Studies in mice

A 3000-μg quantity of NOAC per animal was administered orally to female ICR mice, which were fasted overnight before treatment. The mice were sacrificed after 1, 2, 4, 8, 16, 24, 48 and 72 h. At each time point the blood of three mice was collected in heparin-coated tubes which contained 25 μl of THU solution. Plasma was separated by centrifugation, and the erythrocytes were washed once in PBS and suspended in the corresponding amount of THU solution. The concentration of NOAC in plasma and erythrocytes was determined as described above.

3. Results and discussion

Representative chromatograms for NHAC and NOAC in plasma and erythrocytes of spiked samples are shown in Fig. 2, demonstrating the absence of interfering endogenous substances and baseline separation of both compounds. All peaks were symmetrical and well resolved. The standard curves for NHAC and NOAC were linear in the range of 20–2100 μg/l in plasma and in the range of 40–4200 μg/l in erythrocytes.

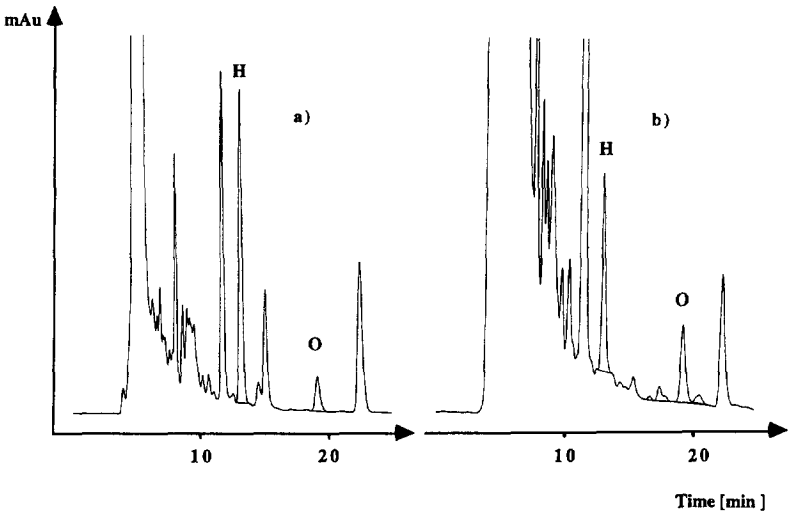

Fig. 2. Chromatograms of standards; the concentration is 500 μg/l NHAC (H) and 625 μg/l NOAC (O), respectively. (a) In plasma: retention time is 13.1 min for NHAC and 19.1 min for NOAC, the detection limit for both analytes is 15 μg/l. (b) In erythrocytes: retention time is 13.0 min for NHAC and 19.3 min for NOAC, the detection limit for both analytes is 30 μg/l.

The linear regression equation for NHAC was $y = 0.0066x + 0.0788$ ($r = 0.999$) in plasma and $y = 0.0014x + 0.1332$ ($r = 0.999$) in erythrocytes. The corresponding values for NOAC were $y = 0.0017x + 0.064$ ($r = 0.999$) in plasma and $y = 0.0014x + 0.1801$ ($r = 0.999$) in erythrocytes. The results of the precision and accuracy experiments are summarised in Table 1. The validation data of both analytes in plasma and in erythrocytes prove that the extraction procedures and the HPLC method were precise and accurate. All between-day and within-day coefficients of variation were below 8.5%. The relative recoveries of NHAC after extraction were 83% for plasma (C.V. 3.6%) and 66.3% for erythrocytes (C.V. 2.1%). For NOAC relative recoveries of 66.3% for plasma (C.V. 5.5%) and 71.4% for erythrocytes (C.V. 9.3%) were determined. It is known that both drugs, which are highly lipophilic, rapidly bind to membranes and proteins, after their exposure to blood [17]. Taking these properties of the analytes into consideration, the rates of recovery ranging from 66% to 83% were satisfying.

When applying the method to blood samples of mice treated orally with NOAC, the amounts of NOAC shown in Fig. 3 were determined. A linear increase of NOAC in plasma during the first 8 h was observed. After 48 h the amount of NOAC in plasma was below the detection limit. In erythrocytes, the corresponding values were significantly higher, which demonstrates the accumulation of NOAC in red blood cells.

The purpose of this study was to establish new analytical methods for the determination of NHAC and NOAC in plasma and erythrocytes

Table 1
Precision and accuracy data for NHAC and NOAC in plasma and erythrocytes

Sample material	Concentration (μg/l)	*n*	Mean (μg/l)	S.D. (μg/l)	C.V. (%)	Accuracy (%)
(a) NHAC						
Plasma	Within-day					
	498	4	496	11.0	2.2	99.6
	1478	4	1457	85.0	5.8	98.6
	Between-day					
	498	4	492	12.8	2.6	98.8
	1478	4	1426	117	8.2	96.5
Erythrocytes	Within-day					
	996	4	1024	51.7	5.0	103
	2956	4	2822	79.1	2.8	95.5
	Between-day					
	996	4	956	13.5	1.4	96.0
	2956	4	2938	73.7	2.5	99.4
(b) NOAC						
Plasma	Within-day					
	500	4	507	28.7	5.7	101
	1484	4	1497	122	8.1	101
	Between-day					
	500	4	515	43.9	8.5	103
	1484	4	1550	85.0	5.5	104
Erythrocytes	Within-day					
	1000	4	893	48.2	5.4	89.3
	2968	4	2410	83.4	3.5	81.2
	Between day					
	1000	4	859	27.1	3.2	85.9
	2968	4	2522	202	8.0	85.0

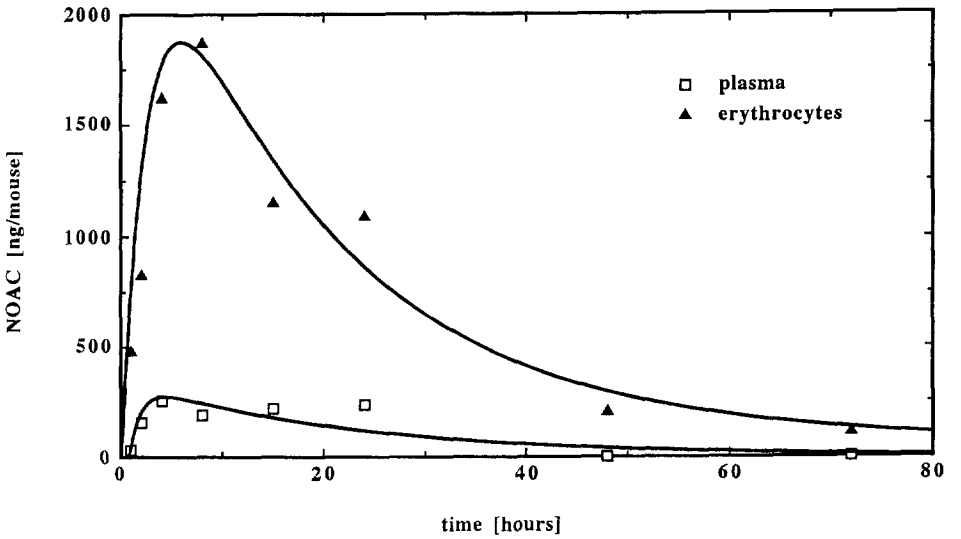

Fig. 3. Pharmacokinetic study in mice after oral application of 3000 μg NOAC (average of 3 mice per timepoint).

by HPLC. These methods should be reproducible and suited for the routine analyses of blood samples of patients undergoing chemotherapy. Since NHAC and NOAC bind strongly to membranes and proteins, the development of an efficient extraction procedure of the drugs from plasma and erythrocytes was of great importance. In plasma the drugs bind predominantly to albumin and the lipoproteins (high-density, low-density and very-low density) [17]. It was found that the treatment of the plasma with organic solvents or perchloric acid co-precipitated the two drugs. Therefore, a concentrated solution of urea (10 *M*) was used for the denaturation of the proteins, which facilitated the release of protein-bound NHAC and NOAC. In erythrocytes the major part of the drugs is integrated into membranes. Urea was not able to destroy the membranes and to solubilize the lipophilic substances. The resulting recoveries from erythrocytes treated with 10 *M* urea were only 4%. Therefore, other procedures had to be developed. The most useful approach was to sonicate the samples, which raised the recoveries to 36% for NHAC and 17% for NOAC. The addition of butanol to the sample before sonication further increased the recoveries to 53% for NHAC and 46% for NOAC; this organic solvent can solubilize the drug after disruption of the membrane. Since the degraded erythrocytes plugged the cartridges when performing SPE, centrifugation or filtration before sample application was required. As a consequence, the recovery rate dramatically decreased, presumably caused by strong binding of the drugs to the erythrocyte membrane debris, as this binding was stronger than their solubility in the butanol–water mixture. Therefore, the cell debris had to be extracted twice, resulting in recoveries greater than 66% for both analytes. Analysing the different fractions of methanol, 8% of NHAC or NOAC was recovered after SPE, 67% in the second fraction, and 25% in the third. Presumably, it should be possible to increase the yield of NHAC and NOAC by repeated extraction of the cell debris with methanol. However, since the procedure is very time-consuming, the "cost/benefit" ratio with respect to time appears to be inappropriate and would compromise an application of the method for routine analysis.

Both drugs analysed have unique chemical structures compared with commonly used other drugs. Therefore, no interference from co-medications is to be expected. Endogenous substances with comparable characteristics to NHAC and NOAC include fatty acids and derivatives of arachidonic acid. These substances are found at higher concentrations in erythrocytes than in the

surrounding aqueous plasma and lead therefore to a chromatogram which is "noisier" compared with plasma. In humans, the individual patterns of these natural lipophilic substances are similar; consequently, the high intercept does not disturb quantification of NHAC and NOAC. The distribution of the lipophilic compounds in erythrocytes of mice differs from that in humans, emphasizing the necessity to calibrate each species individually. For all analyses performed to validate the methods, human blood was used.

The lipophilic properties of the two drugs had to be taken into consideration during the establishment of the HPLC conditions, necessitating an increase in the amount of methanol in the mobile phase to 90%. To separate the peaks of NHAC and NOAC from endogenous peaks, two columns had to be connected in series. This resulted in long retention times and broad peaks. To decrease the retention times and to diminish the width of the peaks, the second column was heated to 45°C.

Only scarce data of pharmacokinetic studies of N^4- or 5′-acyl derivatives of ara-C have been reported in the literature. The HPLC method which was described for the analysis of N^4-behenoyl-1-β-D-arabinofuranosylcytosine (BHAC) uses similar HPLC conditions to those in this report [18]. The extraction from plasma was performed with tetrahydrofuran, an organic solvent which also solubilizes protein-bound drugs but precipitates the proteins. Because the method has not been published in detail, no data on analytical performance and recovery are available. There is no specific analytical method published for the determination of N^4-palmitoyl-1-β-D-arabinofuranosylcytosine (PLAC) [19]. In clinical pharmacokinetic studies with this drug, PLAC was hydrolized to ara-C, which was determined by radioimmunoassay. For the HPLC analysis of 1-β-D-arabinofuranosylcytosine-5′-stearylphosphate, the conditions were again comparable with the method for NHAC and NOAC [20]. Deproteinization was performed with methanol, but again no data on the analytical performance of the method were given.

The effectiveness of THU as a potent inhibitor for the deamination of ara-C to ara-U in vitro and in vivo has been previously shown by several authors [21,22]. The structures and chemical properties of NHAC and NOAC demonstrate their stability against enzymatic and hydrolytic deamination. To exclude any instability of the samples, THU was added as a precautionary measure.

As demonstrated, it is not the HPLC procedure of the lipophilic derivatives of ara-C which was difficult to handle but rather the extraction strategy of the drugs from serum and erythrocytes which had to be optimized and standardized to obtain satisfactory results.

The data of the pilot study in mice demonstrated the need to analyse NHAC and NOAC not only in plasma but also in erythrocytes because of their prominent binding to erythrocyte membranes. They additionally demonstrate that NOAC is well absorbed after oral application. The pharmacokinetic evaluation of these experiments will supply the rationale for dosage and treatment schedules in humans.

In conclusion, we described an extraction procedure for NHAC and NOAC from human plasma and erythrocytes and presented an analytical approach whose performance has been validated with respect to accuracy, reproducibility, and recovery. The validation data are satisfactory, which renders this method suitable for the monitoring of pharmacokinetic studies with these drugs in the clinical setting.

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