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Determination of the novel topoisomerase I inhibitor NU/ICRF 505 and its major metabolite in plasma, tissue and tumour by high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic technique is presented for the determination of the novel topoisomerase I inhibitor NU/ICRF 505 (a tyrosine conjugate of anthraquinone), its major metabolite (NU/ICRF 505/M) and an internal standard (NU/ICRF 513, dihydroxyphenylalanine conjugate). The method uses a reversed-phase (Apex ODS-2) stationary phase and a mobile phase consisting of 0.25 *M* ammonium acetate adjusted to pH 3 with 25% (v/v) trifluoroacetic acid and methanol with gradient elution. Between-day variation in retention times were less than 1% for NU/ICRF 505 and 513 and 2.4% for the metabolite. Selective detection was achieved at a wavelength of 545 nm giving a limit of detection of 2 ng on column and 50 ng/ml after sample preparation for all three components. Chromatograms were free from interfering peaks even at very high detector sensitivity. Sample preparation was based on incubation of biological specimens (0.5 ml plasma or homogenate) with dimethylsulphoxide and acetonitrile at 4°C for 30 min followed by centrifugation. Liver and tumour were homogenised in phosphate buffered saline. Recoveries were consistently high (81.7–106.7% for NU/ICRF 505; 88.7–103.3% for NU/ICRF 513 and 83.7–98.7% for NU/ICRF 505/M) with between day coefficients of variation of normally less than 10%. The method will contribute significantly to the preclinical evaluation of NU/ICRF 505.

Keywords: NU/ICRF 505; Topoisomerase I inhibitor

1. Introduction

The nuclear enzymes DNA topoisomerase (topo) I and II represent two of the most important cellular targets for rational design of new anticancer drugs [1,2]. Anthracenylamino acid conjugates (AAC) are novel chemical structures shown to possess a range of specificities as inhibitors of these enzymes [3]. They also exhibit several properties deemed desirable in an antineoplastic agent. Although AAC are quinone-containing compounds they are not likely to generate free radicals species and cause toxicity by this mechanism due to a strongly negative one electron reduction potential [3]. AAC are growth inhibitory to a number of human and animal cancer cell lines at low micromolar drug concentrations (1–20 μM) [4]. In addition, their in vitro activity correlates closely to the level of expression of topo protein in these cell lines, suggesting a more selective mechanism of action, unlike several clinically

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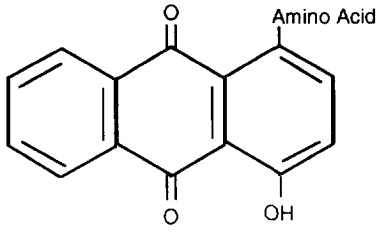
effective but highly toxic topo inhibitors such as doxorubicin, etoposide and ellipticines [5]. Finally, AAC have been demonstrated to be non-cross resistant in cell lines which possess both the classic multidrug resistance (MDR) phenotype and a number of atypical MDR phenotypes related to alterations in topo expression [4].

One of the most promising AAC is the tyrosine conjugate NU/ICRF 505 which inhibits topo I through stabilization of the cleavable complex (for chemical structure see Fig. 1) [3]. NU/ICRF 505 is not recognised by the MDR efflux pump P-170 glycoprotein and is non-cross resistant in a camptothecin resistant Chinese hamster ovary mutant cell line [6,7]. Recently, significant in vivo antitumour activity has been confirmed for NU/ICRF 505 against HT-29 human colon cancer and NX002 human non-small cell lung cancer xenografts [4]. To aid in preclinical evaluation of NU/ICRF 505 as a potential new anticancer drug, a high-performance liquid chromatography assay has been developed for its determination in plasma, normal tissue and xenograft tumour tissue.



Codename	Amino Acid	C-terminus
NU/ICRF 505	Tyrosine	$-COOC_2H_5$
NU/ICRF 505/M	Tyrosine	-COOH
NU/ICRF 513	Dihydroxy-phenylalanine	$-COOCH_3$

Fig. 1. Molecular structures of NU/ICRF 505, 513 and 505/M. For fuller details of these compounds see Section 2.

2. Experimental

2.1. Chemicals and drug standards

All methanol and acetonitrile were HPLC reagent grade and were from Rathburn Chemicals (Walkerburn, UK). Ammonium acetate was HPLC reagent grade and was from FSA Laboratory Supplies (Loughborough, UK) and trifluoroacetic acid was protein sequencing grade obtained from Sigma (Poole, UK). Dimethylsulphoxide (DMSO) was spectroscopic grade and was from NBS Biological (Hatfield, UK). Water was deionised and double distilled in a quartz glass still. All other general chemicals were of the highest grade available commercially and were used as received.

NU/ICRF 505 and NU/ICRF 513 (Fig. 1) were synthesised through the reaction of α-amino acid esters with (2H,3H)-9,10-dihydroxyanthracene-1,4-dione as described in detail (Cummings and Mincher, UK Pat. GB 9205859.3; Int. Appl. No. PCT/GB93/00546, published 30th September 1993). The above procedure results (after oxidation) in mono-substitution of the anthraquinone nucleus uncontaminated by bis-substitution products. The highly crystalline optically pure N-anthracenylamino acid derivatives were characterised by electron impact mass spectrometry, elemental analysis, proton NMR and infrared spectroscopy. They were purified by preparative thin-layer chromatography (TLC), column chromatography and recrystallisation. The major in vivo metabolite of NU/ICRF 505 (the hydrolysis product codenamed NU/ICRF 505/M, Fig. 1) was produced by incubation of the parent drug with mouse plasma. This resulted in 99% conversion into the metabolite which was then purified by column chromatography using reversed-phase C_2 bonded silica (40 μm particle diameter, Bondesil, Varian Sample Preparation, Harbour City, CA, USA). The purified metabolite was characterized by UV–Vis spectroscopy and positive fast atom bombardment mass spectrometry (manuscript in preparation). NU/ICRF 505 and 513 were dissolved and diluted in DMSO and stored refrigerated in crimp capped autosampler vials for no longer than two weeks.

NU/ICRF 505/M was unstable in DMSO and was reconstituted and diluted in methanol and stored refrigerated as above.

2.2. High-performance liquid chromatography

Apparatus consisted of a Hewlett-Packard Model 1090 liquid chromatograph with a diode-array detector (set at 545 nm but without a reference wavelength) configured as reported previously [8]. The stationary phase was Apex ODS-2 prepacked in 25 cm×4.6 mm I.D. stainless steel analytical columns and 1 cm×4.6 mm I.D. stainless steel pre-columns (supplied by Crawford Scientific, Strathaven, UK). The mobile phase consisted of ammonium acetate [0.25 *M*, adjusted to pH 3 with 25% (v/v) trifluoroacetic acid] as buffer A and methanol as solvent B. Gradient elution was employed at a flow-rate of 1 ml/min, at 40°C, using the following linear programme: $t=0$, 40% solvent B; $t=10$ min, 100% solvent B; $t=12$ min, 100% solvent B; $t=16$ min, 40% solvent B. The total run time was 20 min which allowed 4 min for complete re-equilibration of the mobile phase to occur (as determined spectrophotometrically using the diode-array detector).

2.3. Sample preparation

Partially thawed liver and HT-29 human colon cancer xenograft tissue were homogenised in ice cold phosphate buffered saline (PBS, pH 7.4) at 4°C using a Polytron high-speed homogeniser (Northern Media, Hessle, UK) prior to further sample preparation. One gram of liver was added to 4 ml of PBS and whole tumour samples weighing normally between 0.1 and 0.25 g were added to 500 μl PBS. A 0.5-ml volume of homogenate was placed in a 1.5-ml screw-capped polypropylene micro tube (product number 72.692, Starstedt, Leicester, UK). A 0.5-ml volume of human blood bank plasma was used for control sample preparations and a 0.05–0.3 ml plasma volume used for drug analysis from animals treated with NU/ICRF 505. To this was added 100 μl of DMSO and 500 μl of ice cold acetonitrile. In the case of control experiments, the 100 μl of DMSO contained either NU/ICRF 505 or 513 at a variety of different concentrations (see Section 3). NU/ICRF 505/M was added to plasma or homogenates in control experiments in 100 μl of methanol in addition to 100 μl of DMSO and 500 μl of acetonitrile. Tubes were screw-capped and then incubated on ice for 30 min after which time they were centrifuged at 14 000 *g* in a micro-centrifuge (Eppendorf Model 5415C, obtained from Merck, Poole, UK). After centrifugation, the supernatant was decanted and placed into a smoked glass autosampler vial and crimp capped prior to analysis by HPLC as described above. Up to 100 μl of sample could be injected on column.

2.4. Analysis of samples

Animals were dosed with NU/ICRF 505 and plasma, liver and tumour specimens were collected at several different time points. The animal model was the nude mouse (*nu/nu*) purchased from OLAC (Oxford, UK) and they were maintained in negative pressure isolators (La Calhene, Cambridge, UK). The HT-29 human colon xenograft was established in nude mice from the HT-29 cell line and was grown as a subcutaneous tumour in the flank of animals. NU/ICRF 505 was administered intraperitoneally (i.p.) as a 10% DMSO solution in sterile water at a dose of 25 mg/kg. Tumour and liver were also collected from non-drug treated mice to utilize in control experiments and as blanks. After samples were collected (in the case of blood, plasma was first separated) they were immediately placed in liquid nitrogen and stored at −40°C prior to analysis.

3. Results and discussion

3.1. High-performance liquid chromatography

AAC are amphipathic molecules containing a strongly hydrophobic (non-water soluble) anthraquinone chromophore conjugated to a more water soluble amino acid side chain which can be neutral (NU/ICRF 513), negatively charged (NU/ICRF 505/M) or hydrophobic (NU/ICRF 505). The characteristics of the method developed are contained in Table 1 and a chromatogram illustrating the separation of a mixture of standards is shown in Fig. 2. In keeping

Table 1
High-performance liquid chromatography of the novel topoisomerase I inhibitor NU/ICRF 505, its major metabolite NU/ICRF 505/M and internal standard NU/ICRF 513

Codename	Retention time [mean (min)±C.V. (%)][a]	Limit of detection		Calibration curves (range: 5–5000 ng ($y=mx+c$)[b]	Regression correlation (r^2)
		On column (ng)	After extraction (ng/ml)		
NU/ICRF 505	13.2±0.2	2	50	$y=1.18x+13.2$	1.000
NU/ICRF 513	11.9±0.3	2	50	$y=1.45x+17.7$	1.000
NU/ICRF 505/M	11.5±2.4	2	50	$y=1.47x+4.8$	1.000

[a] Between-day coefficient of variation in retention time.
[b] y=concentration, x=integrated peak area, m is the gradient of the calibration curve and c is the intercept with the y-axis.

with related anthraquinoid structures such as mitoxantrone [9,10], ametantrone [11] and CI941 (biantrazole, DUP941) [12], a high ionic strength buffer was required together with a reversed-phase (C_{18}) HPLC column to reduce secondary column interactions and peak tailing. All 3 compounds share almost identical absorption spectra with a broad visible maximum centred around 545 nm, and this wavelength was chosen for highly selective detection free from interference from endogenous substances present in biological specimens. However, a reference wavelength was avoided as this resulted in the introduction of large negative peaks co-eluting with AAC during the analysis of biological samples. By adoption of a gradient elution method, this produced very stable retention times which varied by less than 1% for NU/ICRF 505 and 513 over a two-month period. At the 3:1 ratio of peak signal to background noise, the limit of detection on column was 2 ng for each compound and 50 ng/ml after sample preparation and this was sufficiently sensitive to enable determination of NU/ICRF 505/M at later time points (6 h after drug administration) in preclinical animal pharmacokinetic studies.

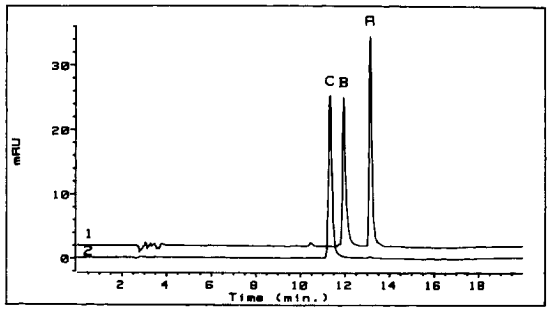


Fig. 2. Reversed-phase HPLC chromatograms of standard solutions of anthracenylamino acids conjugates. Chromatographic details are reported in Section 2. The concentration of each compound was 10 μg/ml, 20 μl were injected on column and peaks were monitored at 545 nm. Chromatogram 1: peak A is NU/ICRF 505 (t_R, 13.1 min) and peak B is NU/ICRF 513 (t_R, 12.0 min). Chromatogram 2: peak A is NU/ICRF 505/M (t_R, 11.3 min)

3.2. Sample preparation

In a preliminary report on the analysis of AAC, a solid-phase extraction technique based on C_2 bonded reversed-phase silica was developed and shown to offer high recoveries from cancer cells grown in 5–10% serum [7]. When this technique was applied to whole plasma and especially tissue homogenates, very low recoveries were achieved (<10%) due to a lack of retention on the solid-phase support. C_8 and C_{18} bonded reversed-phase silica were also investigated using up to 500 mg of sorbent but extraction efficiencies remained poor. Precipitation of proteins prior to solid-phase extraction (SPE) improved yields only slightly but these values were dependent on the protein precipitating agent. Trichloroacetic acid was effective in precipitating proteins but AAC were insoluble in this solvent; AAC were soluble in DMSO but this solvent did not produce efficient precipitation of proteins. It was concluded that SPE was unsuitable and in the respect AAC are different from related anthraquinoids such as mitoxantrone and biantrazole where SPE has been successfully

applied utilising C_2 and C_{18} [10,12]. Biological specimens were finally processed using a microassay where they were incubated with a mixture of ice cold acetonitrile and DMSO to effectively precipitate proteins and maintain the solubility of AAC.

Percentage recoveries obtained using this technique are shown in Table 2. Consistent and high recoveries were achieved with NU/ICRF 505 and its hydrolysis product NU/ICRF 505/M at most concentrations in all the biological matrices studied. Recovery of NU/ICRF 513 which is closely related in structure to NU/ICRF 505 (see Fig. 1) was very similar to NU/ICRF 505, indicating that this compound is a good candidate as an internal standard. Due to the rapid metabolism of NU/ICRF 505 and 513 in mouse liver (and plasma) (Cummings et al., manuscript submitted for publication) data were generated only for NU/ICRF 505/M.

The maximum volume of biological specimen that could be analyzed by this microassay technique was 500 μl and the maximum volume that could be injected on column was 100 μl giving a limit of detection after sample preparation of 50 ng/ml (Table 1). The microassay yielded very clean samples both from the point of view of a lack of interfering peaks on chromatograms (see Fig. 3 for human plasma and Fig. 4 for mouse liver) and from the point of view of analytical column lifetime. In the case of the latter, the same analytical column could be used for several months with precolumns being changed after approximately 30–50 injections of biological specimens.

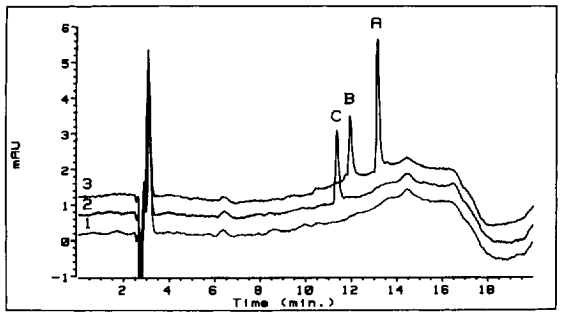


Fig. 3. HPLC analysis of human plasma samples spiked with 1 μg/ml anthracenylamino acid conjugates. Sample preparation was with DMSO and acetonitrile as described in Section 2. Chromatogram 1: blank plasma sample. Chromatogram 2: sample spiked with NU/ICRF 505/M (Peak C, 11.4 min). Chromatogram 3: sample spiked with NU/ICRF 505 (peak A, 13.2 min) and NU/ICRF 513 (peak B, 11.9 min).

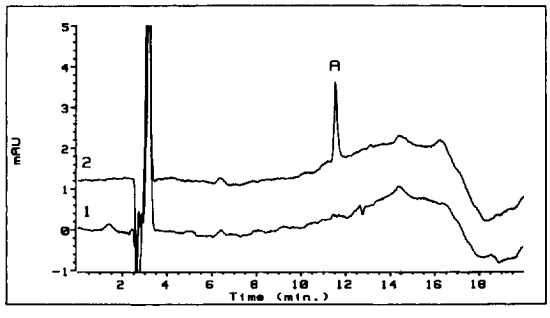


Fig. 4. HPLC analysis of mouse liver homogenate samples spiked with 1 μg/ml NU/ICRF 505/M. Chromatogram 1: blank sample (20%, w/v in phosphate buffered saline). Chromatogram 2: sample spiked with NU/ICRF 505/M (peak A, 11.6 min).

Table 2
Percentage recovery (mean±S.D.) of the novel topoisomerase I inhibitor NU/ICRF 505, its major metabolite NU/ICRF 505/M and internal standard NU/ICRF 513 from plasma, liver and tumour tissue after sample preparation

Sample	Concentration (μg/ml)	NU/ICRF 505	NU/ICRF 513 (internal standard)	NU/ICRF 505/M
Human	0.10	81.7±16.7[a]	88.7±3.9	94.4±12.4
Plasma	1.00	105.3± 4.3	91.6±4.6	83.7± 7.2
	10.0	106.7± 2.0	103.3±2.0	90.4± 2.7
Mouse	0.10	NA[b]	NA	95.4± 4.4
Liver	10.0	NA	NA	98.7± 4.3
HT-29 human colon cancer xenograft	10.0	94.8[d]	77.8	ND[c]

[a] Mean value±between day S.D from $n=5-10$ replicates at each different concentration.
[b] NA, not evaluable due to rapid metabolism of NU/ICRF 505 and 513 by mouse liver.
[c] ND, not determined.
[d] $n=2$.

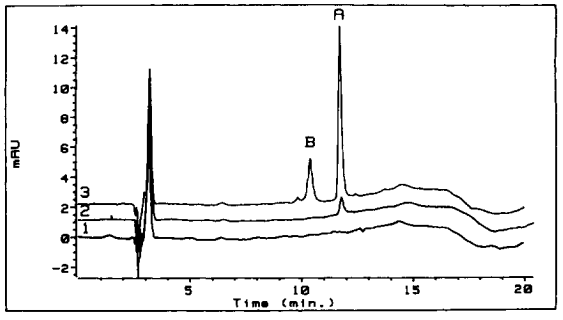


Fig. 5. HPLC analysis of liver and HT-29 human colon cancer xenograft samples collected from a nude mouse 2 h after i.p. injection of NU/ICRF 505 at a dose of 25 mg/kg. HPLC conditions and sample preparation technique are described in full in Section 2. Chromatogram 1:tumour homogenate from a non-drug treated mouse. Chromatogram 2: tumour homogenate from a drug treated mouse, peak A (11.8 min) is NU/ICRF 505/M at a concentration of 4.6 μg/g. Chromatogram 3: liver homogenate from a drug treated mouse, peak A (11.8 min) is NU/ICRF 505/M at a concentration of 17.8 μg/g and peak B is a further unidentified metabolite of NU/ICRF 505. Note that no parent drug was detected in either tissue specimen.

3.3. Analysis of samples

Fig. 5 shows the analysis of a liver and the HT-29 human colon cancer xenograft taken from a nude mouse 2 h after i.p. administration of 25 mg/kg NU/ICRF 505. No intact parent drug was detected in these samples. The only peak detected in tumour and plasma (data not shown) was the metabolite NU/ICRF 505/M (peak A, Fig. 5). In the liver, an additional peak was detected (peak B, Fig. 5) which has subsequently been identified as a glucuronide of NU/ICRF 505/M (Cummings et al., manuscript submitted for publication). These data suggest that NU/ICRF 505 may be a pro-drug.

The above in vivo results were not surprising since during method development, it was evident that murine specimens (plasma and liver) rapidly metabolised both NU/ICRF 505 and 513 in situ into their respective hydrolysis products. This biotransformation did not occur in human specimens, either in fresh plasma taken from volunteers or blood blank plasma, as well as the HT-29 xenograft.

4. Conclusion

In conclusion, a gradient elution HPLC method with a microassay sample preparation technique involving incubation of 500 μl of biological specimens with 100 μl DMSO and 500 μl acetonitrile has been developed for the determination of the novel topo I inhibitor NU/ICRF 505 in plasma, normal tissues and tumour tissue. This method is sufficiently sensitive to be used in pharmacokinetic studies, is capable of identifying important metabolites and will contribute significantly to the preclinical development of this interesting new investigational agent.

References

[1] A.H. Corbett and N. Osheroff, Chem. Res. Toxicol., 6 (1993) 586.
[2] A.Y. Chen and L.F. Liu, Annu. Rev. Pharmacol. Toxicol., 34 (1994) 191.
[3] I. Meikle, J. Cummings, J.S. Macpherson, J.A. Hadfield and J.F. Smyth. Biochem. Pharmacol., 49 (1995) 1747.
[4] J. Cummings, J.S. Macpherson, I. Meikle and J.F. Smyth, Biochem. Pharmacol., (1996) in press.
[5] J. Cummings and J.F. Smyth, Ann. Oncol., 4 (1993) 533.
[6] S.M. Davies, S.L. Davies, A.G. Hall and I.D. Hickson, Mutat. Res., 235 (1990) 111.
[7] J. Cummings, I. Meikle, J.S. Macpherson and J.F. Smyth, Cancer Chemother. Pharmacol., 37 (1995) 103.
[8] J. Cummings, D.J. Kerr, S.B. Kaye and J.F. Smyth, J. Chromatogr., 431 (1988) 77.
[9] F.S. Chiccarelli, J.A. Morrison, D.B. Cosulich, N.A. Perkinson, D.N. Ridge, F.W. Sum, K.C. Murdock, D.L. Woodward and E.T. Arnold, Cancer Res., 46 (1986) 4858.
[10] Y-M. Peng, D. Ormberg and D.S. Alberts, J. Chromatogr., 233 (1982) 235.
[11] J.G. Kuhn, C.E. Balmer, T.M. Ludden, D.M. Loesch, D.D. Von Hoff, J.F. Bender and A.J. Grillo-Lopez, Cancer Chemother. Pharmacol., 19 1987 133.
[12] M.A. Graham, D.R. Newell and A.H. Calvert, J. Chromatogr., 491 1989 253.