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Short communication

Determination of FCE 26644, a new polysulphonated derivative of distamycin A, in monkey plasma by reversed-phase ion-pair high-performance liquid chromatography with ultraviolet detection

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Abstract

A sensitive and selective ion-pair high-performance liquid chromatographic method for the determination of 7,7′-{carbonylbis[imino-*N*-methyl-4,2-pyrrolecarbonylimino(*N*-methyl-4,2-pyrrole)carbonylimino]}-bis(1,3-naphthalenedisulphonic acid), tetrasodium salt in monkey plasma has been developed. The compound and internal standard (bromphenol blue) were extracted from plasma samples with methylene chloride (twice) after deproteination with acetonitrile and addition of the ion-pairing agent (tetrabutylammonium hydroxide). The combined organic phases were dried, the residue dissolved in the mobile phase and then analysed by reversed-phase ion-pair liquid chromatography under isocratic conditions. The HPLC analysis time was about 20 min. Quantitation was achieved by UV detection at 323 nm. The linearity, precision and accuracy of the method were evaluated. The limit of quantitation was 0.3 μg/ml plasma. No interference from blank monkey, mouse, rat, dog and human plasma was observed. The suitability of the method for in vivo samples was checked by analysis of plasma samples drawn from three male cynomolgus monkeys that had received a 20 mg/kg single i.v. dose of the test compound.

Keywords: Distamycin A derivative; 7,7′-{Carbonylbis[imino-*N*-methyl-4,2-pyrrolecarbonylimino(*N*-methyl-4,2-pyrrole)carbonylimino]}-bis(1,3-naphthalenedisulphonic acid)

1. Introduction

The evidence that solid tumours are angiogenesis-dependent [1] suggested the therapeutic use of compounds able to inhibit the neo-vascularization induced by angiogenic factors [e.g. basic fibroblastic growth factor (bFGF), platelet-derived growth factor (PDGFβ)] produced by the tumour itself. Suramin, a polysulphonated naphthylurea previously used against trypanosomiasis and onchocerciasis, has more recently been shown to have potential value in the treatment of some solid tumours and in patients with HTLV-III/LAV infection, the etiologic agent of acquired immune deficiency syndrome (AIDS) [2–6]. This drug, however, shows a very narrow therapeutic index and several dose-limiting toxic effects which require careful monitoring of plasma levels during therapy [7].

In a research program aimed at developing pharmacological agents able to inhibit tumour angiogenesis and endowed with lower toxicity than suramin, a number of novel polysulphonated deriva-

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tives of distamycin A (see Fig. 1), were synthesized [8]. Among these is 7,7′-{carbonylbis[imino-*N*-methyl - 4,2 - pyrrolecarbonylimino(*N* - methyl - 4,2 - pyrrole)carbonylimino]} - bis(1,3 - naphthalenedisulphonic acid), tetrasodium salt (Laboratory code FCE 26644, I, see Fig. 1), a new drug currently under investigation as an inhibitor of angiogenesis. This compound was found to be active in some in vitro and in vivo models, being able to inhibit angiogenesis in the chorioallantoic membrane assay and to bind bFGF and PDGFβ [9,10]. In addition, it was found to inhibit in mice the growth of i.m. implanted M5076 murine reticulosarcoma at non-toxic doses with a 50% increase in survival time [10].

Due to structural analogies (see Fig. 1), I might have pharmacokinetic characteristics similar to those observed for suramin. In humans suramin was shown to be eliminated very slowly from plasma with terminal half-lives ranging from 44 to 54 days and appeared to be highly bound to plasma proteins [6]. Determination of suramin in plasma by high-performance liquid chromatography (HPLC) has been described [11–16]. The assays are based on reversed-phase ion-pair HPLC, one of them involving gradient elution and the others isocratic separation.

This paper describes the determination of I by ion-pair HPLC with isocratic elution after ion-pairing extraction of the analyte from plasma. The HPLC method developed was fully validated down to a concentration of 0.3 μg/ml in monkey plasma and employed for the quantitation of the drug in plasma samples obtained from cynomolgus monkeys that had received a 20 mg/kg single i.v. dose of the test compound.

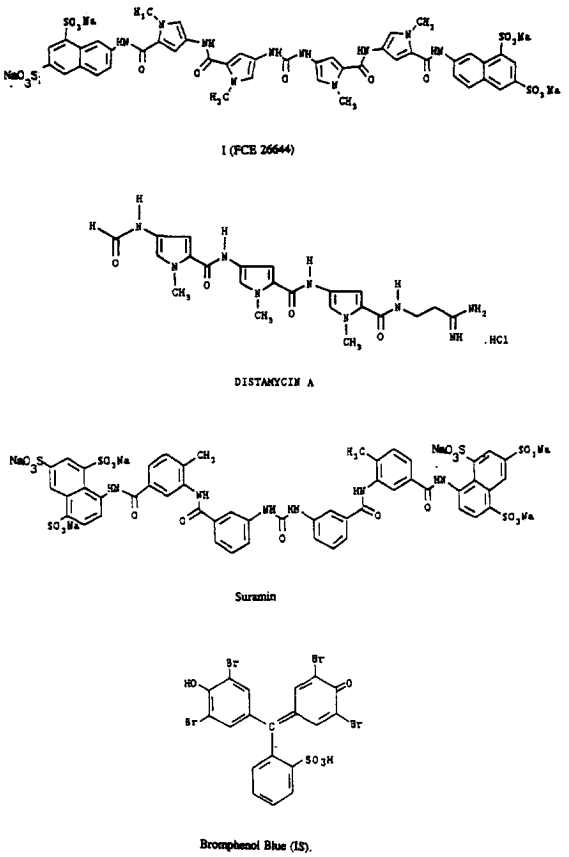

Fig. 1. Structural formulae of I (FCE 26644), distamycin A, suramin and Bromphenol blue (I.S.).

2. Experimental

2.1. Chemicals and solutions

I (as tetrasodium salt) was supplied by the Chemistry Department of Pharmacia S.p.A. All other chemicals and solvents were of analytical reagent grade from Carlo Erba Reagents (Milan, Italy) with the exception of 40% (1.5 *M*) tetrabutylammonium hydroxide (TBAOH) which was purchased from Fluka (Buchs, Switzerland).

Stock solutions were prepared by dissolving a weighed amount of I in bidistilled water and Bromphenol blue (I.S.) (see Fig. 1) in methanol. From these solutions, which are stable for at least 1 month if stored at 4°C, working solutions were prepared every 2 weeks by dilution with bidistilled water. Stock and working solutions were stored a 4°C until use.

All glassware was silanized before use by treatment with 7% dimethyldichlorosilane in toluene solution followed by rinsing with ethanol, to minimize adsorption.

2.2. Equipment

The HPLC system used in this study consisted of an isocratic pump (Model Isochrom), an autosampler

(Model AS 1000) with a 200 μl loop, a variable wavelength UV detector (Model Spectro Monitor 3200) and a data acquisition system (Model Winner 386) with Labnet software. Real-time chromatograms were obtained on an integrator (Model Chromjet). All these instruments were supplied by Thermo Separation Products (San Jose, CA, USA).

2.3. Chromatographic conditions

The chromatographic separation was performed with a 150 mm × 3.9 mm I.D. Nova-Pak C_{18} reversed-phase column (particle size 4 μm) (Waters, Milford, MA, USA) with a Survival precolumn packed with pellicular ODS (particle size 30–38 μm) (Whatman, Clifton, NJ, USA). The mobile phase consisted of a 0.05 *M* monobasic potassium phosphate–acetonitrile mixture (56:44, v/v) containing the ion-pairing reagent TBAOH (0.060 *M*) buffered to pH 7.0 with 85% orthophosphoric acid. The flow-rate was 0.5 ml/min. The detector was set at 323 nm and wired to send a 1 V/AUFS signal to the data system.

2.4. Sample extraction procedure

A 1.0-ml aliquot of the plasma sample was placed in a glass-stoppered test-tube and 0.1 ml of working I.S. solution (about 10 μg) was added. To this solution the following were added in sequence: 1 ml of acetonitrile (vortex mixing for 30 s in order to precipitate plasma proteins), 1 ml of water, 0.1 ml of 0.4 *M* TBAOH (vortex mixing for 15 s) and 5 ml of methylene chloride. The mixture was shaken by a mechanical stirrer for 15 min and after centrifugation (10 min at 1200 *g*) the lower organic phase was removed and retained. A second aliquot of methylene chloride (4 ml) was added to the aqueous phase and the extraction procedure was repeated. The organic phases were combined and evaporated to dryness under a stream of nitrogen (at 37°C). The residue was dissolved in the mobile phase (from 1 to 6 ml according to the expected sample concentration) by vortex-mixing for 15 s.

The solution obtained was washed with 2 ml of *n*-hexane (vortex mixer for 30 s) and, after centrifugation, the upper organic phase was discarded. The aqueous phase was then transferred into a plastic vial in the autosampler and an aliquot (200 μl) was injected onto the column.

2.5. Determination of I concentration in quality control and calibration samples.

Analyses of blank monkey plasma spiked with known amounts of I and I.S. were carried out applying the above procedure. The linearity was evaluated from five calibration curves prepared and run on five different days in the concentration range 0.3–100 μg/ml plasma (concentrations were expressed as free acid). The precision and accuracy were evaluated by repeated analyses of I at three concentrations (about 0.8, 5 and 50 μg/ml) in three replicate samples analysed on five different days. All chromatograms obtained were evaluated by peak-area measurement. The concentration in quality control samples was calculated using the calibration curve generated on each day by linear regression (weighting factor $1/y^2$) of the analyte/I.S. peak-area ratio against their concentration ratio in plasma.

To evaluate the extraction recovery, the peak area of extracted plasma samples was compared with the peak area obtained with standards dissolved in the mobile phase and injected directly onto the chromatograph.

2.6. Chromatographic system suitability test

The suitability of the chromatographic system for the analysis of I was checked during the validation assay by calculating the column efficiency, the peak symmetry and the resolution factor of the peaks of the two analytes. This evaluation was carried out according to USP [17] using the System Suitability Test software supplied by Thermo Separation Products.

The column efficiency was expressed as the number of theoretical plates (*N*). This value must be higher than 2000 for both analytes. The tailing factor (*T*) must be less than 2.2 for both analytes. The resolution factor (*R*) between the peaks of the two analytes must be higher than 2.0.

3. Results

The chromatograms obtained from a standard solution of I and I.S. (A), from blank monkey plasma (B) and from spiked monkey plasma (C) are shown in Fig. 2. The retention times of I and I.S. were about 12 min and 18 min, respectively.

Sharp and symmetric peaks were obtained for I and I.S. under the chromatographic conditions chosen. Isocratic conditions for elution from the chromatographic column were preferred to gradient elution because of the better reproducibility of the separation and the longer life span of the column achieved after several weeks of repeated use. Under these conditions, the analysis time was sufficiently short to allow several samples per day to be assayed. The mobile phase composition was chosen after careful examination of the influence of each constituent on the chromatographic separation obtained. In particular, the concentration of the ion-pairing agent was found to be important for the reproducibility of the separation. Dolan [18] generally recommended a concentration of the ion-pairing agent higher than 25 m*M* and injection of the analytes dissolved in the mobile phase. In our hands the optimal TBAOH concentration in the mobile phase for elution of I and I.S. from the chromatographic column was 60 m*M*.

The silanization of the glassware before use and the addition of acetonitrile to the plasma sample effectively improved the extent as well as the reproducibility of the extraction step. The optimal amount of added ion-pairing agent for extraction of I from plasma was 40 μmol. Interestingly, Supko et al. reported a similar value for the assay of suramin [16].

This procedure involves minimal extraction of endogenous compounds from the complex plasma matrix and none of them was found to interfere with the compounds of interest. Mouse, rat, dog and human plasma can also be assayed by this procedure since the chromatograms obtained were free from interfering peaks at the retention times of the compounds of interest.

The extraction recovery of I from monkey plasma in the concentration range 0.3–100 μg/ml ranged from 59.2 to 73.0% (see Table 1). Bromphenol blue showed good performances under the chromatographic conditions employed for analysis of I. The extraction recovery of Bromphenol blue tested at a concentration of about 10 μg/ml of monkey plasma was 68.3 ± 2.1% (mean ± S.D., $n = 5$); it was therefore considered suitable as I.S. in the assay.

The linearity of this HPLC assay was evaluated from five calibration curves run on different days in the concentration range 0.3–100 μg/ml monkey

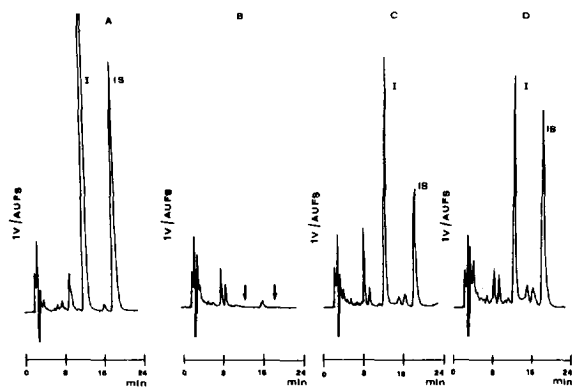

Fig. 2. Chromatogram of (A) standard solution containing 10.03 μg/ml of I and 13.12 μg/ml of I.S., (B) blank monkey plasma (1 ml) (the arrows show where I and I.S. are eluted), (C) monkey plasma (1 ml) spiked with 8.35 μg of I and 13.12 μg of I.S. and (D) plasma (1 ml) from a monkey given a 20 mg/kg i.v. dose of I (48 h after administration). Peaks: I, FCE 26644; I.S., Bromphenol blue.

Table 1
Extraction recovery of I and I.S. from monkey plasma

Label concentration in plasma (μg/ml)	Recovery (%)
Extraction recovery of I from monkey plasma	
0.3	59.2
2.0	62.7
10.0	61.6
30.0	73.0
100.0	69.3
Extraction recovery of I.S. from monkey plasma	
10	68.6
10	68.4
10	69.2
10	70.3
10	64.8
Mean ± S.D. ($n = 5$)	68.3 ± 2.1

plasma. A linear regression analysis of the peak area ratio (analyte/I.S.) against the concentration ratio (analyte/I.S.) showed good linearity over the whole range of concentrations tested.

The mean calibration curve obtained was described by the equation $y = 3.3939x - 0.0154$ (slope C.V. 4.0%, $n = 5$). Back-calculated concentrations exhibited a C.V. of less than 4.9%. Correlation coefficients (r) ranged from 0.9906 to 0.9962.

In toxicity and clinical studies plasma levels of I might be higher than 100 μg/ml, for example at steady-state conditions. Therefore if the concentration of an unknown sample is suspected to fall outside the range of linearity of the calibration curve, the sample may be assayed using a lower volume of plasma made up to 1 ml with blank plasma.

The inter-day precision for concentrations between 0.8 and 50 μg/ml of monkey plasma expressed as C.V. ranged from 8.78 to 12.38% (see Table 2). At the same concentrations, the intra-day precision was better than 11.89%. The intra-day accuracy, evaluated on the same plasma samples and expressed as percentage ratio of the mean amount found to the amount added to plasma, ranged from 82.08 to 115.42%. The pooled accuracy (inter-day) over the 5-day validation period ranged from 99.17 to 105.10%.

The limit of quantitation (LOQ), chosen as the lowest point on the calibration graph having a back-calculated concentration within 20% of the nominal value, was 0.3 μg/ml plasma.

The stability of I in monkey and dog plasma spiked samples (at concentrations of about 2 and 100 μg/ml) stored for 3, 6 and 12 months at −20°C and then assayed by the present HPLC method was studied. In addition the stability of I in spiked human blood samples (at the concentration of about 20 μg/ml) stored for 2 h at room temperature and at +4°C was also studied. Under all these conditions the compound proved to be stable. The compound was also found to be stable when stored dissolved in the mobile phase for at least 48 h at room temperature in plastic vials, thus allowing the use of a non-refrigerated autosampler for automatic injection onto the column of the extracted samples.

The present method was applied to the determination of plasma levels of I in three male cynomolgus monkeys who had received a single i.v. 20 mg/kg dose of I as a slow bolus injection over a period of about 5 min. The animals were between 4 and 6 months of age and weighed between 2 and 3 kg. Blood samples were drawn into heparinized tubes at 0, 5, 15, 30 min, 1, 2, 4, 6, 24, 48, and 96 h and then 7, 10, 14, 21, 28, 35, 42, 49, 55 and 60 days

Table 2
Accuracy and precision of the method for the determination of I in monkey plasma

Control sample (μg/ml)	Day	n	Accuracy			Precision		
			Mean found (μg/ml)	Mean recovery (intra-day) (%)	Pooled recovery (inter-day) (%)	S.D.	% C.V. (intra-day)	Pooled % C.V. (inter-day)
0.80	1	3	0.77	95.83		0.06	7.18	
	2	3	0.66	82.08		0.02	2.33	
	3	3	0.92	115.42		0.01	0.63	
	4	3	0.84	105.42		0.07	8.41	
	5	3	0.78	97.08	99.17	0.03	3.24	12.38
4.98	1	3	4.54	91.10		0.42	9.17	
	2	3	4.77	95.78		0.21	4.36	
	3	3	5.69	114.26		0.20	3.53	
	4	3	5.16	103.55		0.61	11.89	
	5	3	5.05	101.34	101.20	0.19	3.75	10.09
49.81	1	3	50.84	102.07		4.14	8.15	
	2	3	45.71	91.76		1.27	2.79	
	3	3	56.60	113.64		1.11	1.97	
	4	3	52.76	105.92		1.42	2.70	
	5	3	55.83	112.09	105.10	3.25	5.83	8.78

after dosing. The samples were immediately centrifuged at 1200 *g* for 15 min at room temperature. The plasma separated was stored at −20°C until assayed. A typical chromatogram obtained from one monkey at 48 h after dosing is shown in Fig. 2D. Mean ± S.D. plasma concentrations decreased from 382.6 ± 89.7 μg/ml at 5 min to 0.85 ± 0.07 μg/ml at 60 days after dosing.

4. Conclusions

The method described here is sensitive and selective for the determination of I in monkey plasma. It proved to be linear, precise and capable of accurately quantitating the analyte in the concentration range of 0.3–100 μg/ml. The suitability of this method was demonstrated in a pharmacokinetic study of I in cynomolgus monkeys. Therefore this method can be very useful in further investigations on the pharmacokinetics of I in preclinical and clinical studies carried out with the test compound.

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References

[1] J. Folkman. J. Natl. Cancer Inst., 82 (1990) 4–6.
[2] C.A. Stein, R.V. LaRocca, R. Thomas, N. McAtee and C.E. Myers, J. Clin. Oncol., 7 (1989) 499–508.
[3] M.A. Eisenberger, L.M. Reyno, D. Jodrell, V.J. Sinibaldi, K.H. Tkaczuk, R. Sridhara, E.G. Zuhowski, M.H. Lowitt, S.C. Jacobs and M.J. Egorin, J. Natl. Cancer Inst., 85 (1993) 611–621.
[4] R.J. Motzer, D.M. Nanus, P. O'Moore, H.I. Scher, D.F. Bajorin, V. Reuter, W.P. Tong, J. Iversen, C. Louison, A.P. Albino and G.J. Bosl, Cancer Res., 52 (1992) 5775–5779.
[5] B.D. Cheson, A.M. Levine, D. Mildvan, L.D. Kaplan, P. Wolfe, A. Rios, J.E. Groopman, P. Gill, P.A. Volberding, B.J. Poiesz, M.S. Gottlieb, H. Holden, D.J. Volsky, S.S. Silver and M.J. Hawkins, J. Am. Med. Assoc., 258 (1987) 1347–1351.
[6] J.M. Collins, R.W. Klecker, R. Yarchoan, H.C. Lane, A.S. Fauci, R.R. Redfield, S. Broder and C.E. Myers, J. Clin. Pharmacol., 26 (1986) 22–26.
[7] H.I. Scher, D.I. Jodrell, J.M. Iversen, T. Curley, W. Tong, M.J. Egorin and A. Forrest, Cancer Res., 52 (1992) 64–70.
[8] M. Ciomei, W. Pastori, M. Mariani, F. Sola, M. Grandi and N. Mongelli, Biochem. Pharmacol., 47 (1994) 295–302.
[9] M. Mariani, A. Paio, M. Ciomei, W. Pastori, C. Franzetti, G. Melegaro, M. Grandi and N. Mongelli, in R. Steiner, P.B. Weisz and R. Langer (Editors), Angiogenesis: Key Principles–Science–Technology–Medicine, Birkhauser Verlag, Basel, Vol. 61, 1992, pp. 455–458.
[10] F. Sola, G. Biasoli, E. Pesenti, M. Farao, P. Della Torre, N. Mongelli, and M. Grandi, in R. Steiner, P.B. Weisz and R. Langer (Editors), Angiogenesis: Key Principles–Science–Technology–Medicine, Birkhauser Verlag, Basel, Vol. 61, 1992, pp. 459–462.
[11] R.W. Klecker, Jr. and J.M. Collins, J. Liq. Chromatogr., 8 (1985) 1685–1696.
[12] G.Edwards, C.L. Rodick, S.A. Ward, K. Awadzi, M. L'E Orme and A.M. Breckenridge, J. Chromatogr., 343 (1985) 224–228.
[13] R.M. Ruprecht, J. Lorsch and D.H. Trites, J. Chromatogr., 378 (1986) 498–502.
[14] W.P. Tong, H.I. Scher, D.P. Petrylak, A. Dnistrian, T. Curley and J. Vasquez, J. Liq. Chromatogr., 13 (1990) 2269–2284.
[15] U.R. Tjaden, H.J.E.M. Reeuwijk and J. Van Der Greef, J. Chromatogr., 525 (1990) 141–149.
[16] J.G. Supko and L. Malspeis, J. Liq. Chromatogr., 13 (1990) 727–741.
[17] US Pharmacopoeia XXII, Pharmacopoeial Convention, Rockville, MD, 1990, pp. 1566–1567.
[18] J.W. Dolan, LC·GC Int., 5 (N.11) (1992) 16–18.