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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF A 21-AMINOSTEROID ANTIOXIDANT IN PLASMA

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SUMMARY

A novel 21-aminosteroid, 21-[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]-16α-methylpregna-1,4,9(11)-triene-3,20-dione monomethanesulfonate (I), is under development for the treatment of central nervous system injury in humans. This report describes a reversed-phase high-performance liquid chromatographic (RP-HPLC) method using ultraviolet detection at 254 nm for the determination of I in plasma with low nanogram per milliliter sensitivity. Plasma was deproteinated by mixing with acetonitrile and centrifuging, and I was extracted from the supernatant with disposable bonded-phase columns. The extracts were chromatographed on an octylsilane bonded-phase HPLC column with an acetonitrile–water mobile phase containing triethylammonium acetate, pH 5. Quantification was accomplished by peak-height ratio analysis using an analogue of I as the assay internal standard. The method was suitable for the determination of I following a 30 mg/kg intraperitoneal dose in the rat.

INTRODUCTION

Compound I (Fig. 1) is a 21-aminosteroid antioxidant which inhibits lipid peroxidation [1] and is active in models of head and spinal cord injury [2–4]. It has multiple aromatic and aliphatic amine groups and is retained on bonded-phase silica columns in reversed-phase high-performance liquid chromatography (RP-HPLC) by a combination of solvophobic and silanophilic retention mechanisms [5]. The latter predominate unless amine modifier, sodium salt, or acid are added to the mobile phase [5]. The rentention time of I in these systems is therefore easily adjustable. The present report describes a relatively simple HPLC method for the determination of I in plasma which exploits the irregular retention properties of the drug to achieve specificity. The assay was validated for rat plasma, and its utility was demonstrated with a rat intraperitoneal dosing study.

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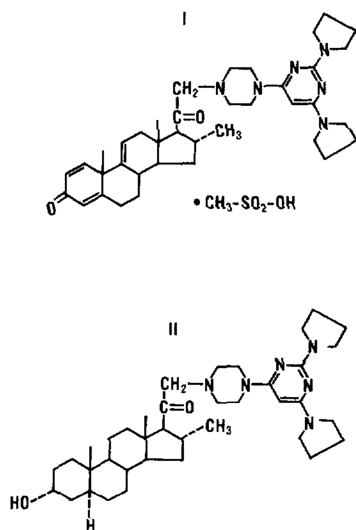

Fig. 1. Chemical structures of 21-[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]-16α-methylpregna-1,4,9(11)-triene-3,20-dione monomethanesulfonate (I) and 21-[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]-3α-hydroxy-16α-methyl-5α-pregnan-20-one (the internal standard, II).

EXPERIMENTAL

Chemicals

Compounds I and II (Fig. 1), both greater than 98% pure, were supplied by the Research and Development Division of the Upjohn Company (Kalamazoo, MI, U.S.A.)*. Triethylamine (TEA) was obtained from Aldrich (Milwaukee, WI, U.S.A.). Acetonitrile, water, and methanol were UV or HPLC grade from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Glacial acetic acid was purchased from Mallinckrodt (Paris, KY, U.S.A.).

Plasma standards

Rat plasma was obtained in-house using sodium heparin or ethylenediaminetetraacetic acid as the anticoagulant. Solution standards of I ranging from 1 mg/ml to 1 μg/ml were prepared in acetonitrile using polypropylene tubes and pipettors. Plasma standards and quality control (QC) specimens were prepared from fresh solution standards by adding 7 μl of the solution standard to 0.5 ml of plasma in a polypropylene tube and vortexing for 1 min. Five plasma standards were prepared at concentrations ranging from 0.012 to 1.5 μg/ml. QC specimens were prepared in advance and stored at $-20\,^{\circ}$C until analysis.

Plasma extraction

Plasma samples (0.5 ml) were diluted with 0.5 ml of acetonitrile containing the internal standard (compound II) (2.5 μg), vortexed for 1 min, and centri-

*The Upjohn Company identification number for I is U-74006F and for II is U-76824.

fuged at 1500 *g* at 4°C to pellet proteins. Bond Elut C_{18} columns (100 mg/ml, Analytichem International, Harbor City, CA, U.S.A.) were mounted on a vacuum manifold (J.T. Baker, Phillipsburg, NJ, U.S.A.) and preconditioned by washing with 2 ml of methanol followed by 2 ml of water. The supernatants (0.8 ml) were applied and drawn through the columns with negative manifold pressure (660 mmHg internal pressure). The columns were washed with 2×1 ml of acetonitrile–water (1:1, v/v) and I was eluted with 2×1 ml of methanol into polypropylene tubes. The methanol eluent was evaporated to dryness at 40°C under a stream of nitrogen. The residue was reconstituted by vortexing for 1 min with 0.35 ml of acetonitrile and then diluted with 0.15 ml of water and transferred to glass vials for HPLC injection.

HPLC instrumentation and conditions

Samples were injected onto the HPLC system using a MicroAS autosampler (Upjohn, not commercially available) equipped with a 100-μl sample loop. The analytical pump was a Beckman 114 solvent delivery module (Beckman Instruments, Berkeley, CA, U.S.A.). The mobile phase was acetonitrile–22 m*M* aqueous TEA adjusted to pH 5.0 with glacial acetic acid (75:25, v/v), and the flow-rate was 1.0 ml/min. The guard column was a NewGuard RP-8, 7 μm particle size, 15×3.2 mm I.D. cartridge (Brownlee Labs., Santa Clara, CA, U.S.A.). The analytical column was a Supelcosil LC-8, 5 μm particle size, 250 mm×4.6 mm I.D. (Supelco, Bellefonte, PA, U.S.A.). Column eluent was monitored at 254 nm with a UV III Model 1203 monitor equipped with an Hg lamp (Laboratory Data Control, Riviera Beach, FL, U.S.A.). The detector output was recorded on a strip chart recorder and collected using an Upjohn automated chromatography system on a Harris 1000 computer (Harris, Fort Lauderdale, FL, U.S.A.).

Quantification method and data analysis

Plasma standard sets consisted of duplicate standards at five concentrations (15, 2, 0.4, 0.07 and 0.01 μg/ml) spanning the assay linear range. Relative weight response factors (RWRFs) for the standards were calculated as the peak height ratio of I to II multiplied by the concentration ratio of II to I. The concentration of unknowns was calculated by dividing the sample peak-height ratio by the mean RWRF for the standard set and multiplying by the concentration of II. Testing for equality of means was by one-way analysis of variance and testing for equality of variances was by Bartlett's test [6].

In vivo studies

Upjohn Sprague–Dawley female rats (220 g) received a single 30 mg/kg intraperitoneal injection of I in 0.05 *M* hydrochloric acid. Blood samples were obtained by serial sacrifice at 2, 4, 8, and 24 h post-dose. Plasma was collected and stored at −20°C.

RESULTS

Plasma extraction

When 0.5 ml of human plasma containing 2 μg/ml I was applied to disposable octadecylsilane bonded-phase columns, approximately 50% was unretained or eluted with a water wash as determined by HPLC analysis against solution standards. In order to test the hypothesis that the poor extractability of the drug from plasma was due to protein binding, plasma proteins were precipitated by mixing with an equal volume of acetonitrile and centrifuging. When the supernatant was applied to the column, less than 10% of the drug was unretained and approximately 85% could be recovered by eluting the column with methanol. It therefore appeared that the drug was protein-bound and that the binding interfered with stationary phase interactions. Interestingly, when a solution of the drug in acetonitrile–water (1:1) was applied to the columns, the retention was quantitative, but only 5% could be recovered by eluting with methanol. Previous work had shown that I was strongly bound by silanol groups on bonded-phase silica packings unless amine modifiers were added to the eluting solvent [5], and it was therefore concluded in this case that non-protein plasma components acted as silanol group deactivating agents.

Based on these results, a plasma extraction procedure was developed that required deproteination of 0.5 ml of plasma by mixing with 0.5 ml of acetonitrile followed by centrifugation. The supernatant (0.8 ml) was applied to the extraction columns, and, after washing with acetonitrile–water (1:1), compound I was eluted with methanol. The overall extraction recovery (including the transfer loss) was 67.8 ± 1.2% (mean ± S.D.) over a concentration range of 0.05–20 μg/ml based on comparison of the RWRF of three standard curve sets with the response factor of solution standards. The extraction recovery of II, the analogue of I used as the assay internal standard, was 64%. The time for one person to extract 60 plasma samples by this procedure, evaporate the methanol under nitrogen, and reconstitute in injection solvent for HPLC analysis was 8 h.

High-performance liquid chromatography

The concentration of I in plasma extracts was determined with an isocratic reversed-phase system employing an end-capped octylsilane column (Supelco LC-8, 5 μm particle size, 250 mm × 4.6 mm I.D.) and UV detection at 254 nm. As a starting point for the development of the assay, the mobile phase composition was acetonitrile–36 m*M* aqueous TEA adjusted to pH 5.0 with acetic acid (7:3, v/v). The capacity factor of I was 3.9. Several cartridge guard columns were tested for compatibility with this system. As shown in Table I, column efficiency was highest with the RP-8 NewGuard, a 7-μm packing material with column dimensions 15 mm × 3.2 mm I.D., which is in agreement with the findings of Lundanes et al. [7] that losses in column efficiency can be minimized by reducing the size and retentiveness of the guard column.

Under these conditions, extracts of human plasma contained interferences in the retention window of I with the largest peak eluting on the tail of the analyte. The baseline in the region after this peak was free of interferences. Compound I

TABLE I

INFLUENCE OF THE GUARD COLUMN ON THE CHROMATOGRAPHIC BEHAVIOUR OF I

Analytical column: Supelcosil LC-8, 250 mm×4.6 mm I.D., 5 μm particle size; mobile phase conditions: acetonitrile–36 m*M* aqueous TEA pH 5.0 (7:3, v/v), flow-rate: 1 ml/min.

Guard column	Retention time of I (min)	Peak height (mm)	Asymmetry factor
None	10.6	161	1.2
RP-8/Spheri-5*	13.2	78	2.3
RP-2/Spheri-5*	11.2	140	1.5
RP-8/NewGuard**	10.7	159	1.3
Phenyl/NewGuard**	10.6	160	1.4

*Spheri column dimensions: 30 mm×4.6 mm I.D., 5 μm particle size.
**NewGuard column dimensions: 15 mm×3.2 mm I.D., 7 μm particle size.

was selectively shifted into this window by decreasing the TEA concentration from 36 to 22 m*M*, which resulted in an increased silanophilic capacity factor with a consequent increase in retention time. All of the peaks were then non-selectively moved towards the solvent front by increasing the acetonitrile concentration from 70 to 75%. The net effect was to move the interference peaks toward the solvent front and away from I while holding the retention time of I constant.

Because of the retention characteristics of I, it was necessary to adjust the mobile phase composition when a new analytical column was installed. For example, a recently manufactured Supelcosil LC-8 column required a mobile phase composition of acetonitrile–35 m*M* aqueous TEA pH 5.0 (84:16, v/v) in order to hold the capacity factor for I constant and avoid plasma interferences. HPLC conditions suitable for human plasma analysis were also suitable for dog and rat plasma analysis.

Despite the sensitivity of the retention time of I to column identity and mobile phase conditions, the chromatography system was sufficiently rugged for routine bioanalytical applications. There was less than a 5% change in the retention time of I between different batches of mobile phase, and the intra-day relative standard deviation (R.S.D.) of the retention time of I for 40 consecutive injections of plasma extracts was less than 0.3%. Analysis of ca. 400 plasma extracts over one month resulted in an increase in column back-pressure of only 1 bar and less than a 10% change in the retention time of I. The retention times of I and II were approximately 10.5 and 16 min, respectively, and the chromatography run time was 18 min.

Detection

Detection was accomplished by measuring UV absorbance at 254 nm with a mercury line source detector. In HPLC mobile phase, I had absorption maxima at 234 nm (ϵ=52 000) and 285 nm (ϵ=17 000). At 254 nm, the extinction coefficient was 22 000. Detection at 229 nm with a cadmium lamp was considered as

TABLE II

RESULTS FROM ANALYSIS OF THREE INDEPENDENTLY PREPARED RAT I PLASMA STANDARD SETS

Plasma concentration (µg/ml)	Relative weight response factor*					R.S.D. (%)
	Standard set 1	Standard set 2	Standard set 3	Mean	S.D.	
0.0120	2.60 (+)	2.41 (−)	2.50 (+)	2.59	0.14	5.5
0.0120	2.74 (+)	2.76 (+)	2.52 (+)			
0.0725	2.37 (−)	2.30 (−)	2.40 (−)	2.43	0.10	4.0
0.0725	2.58 (+)	2.50 (+)	2.41 (−)			
0.425	2.36 (−)	2.44 (−)	2.38 (−)	2.41	0.03	1.3
0.425	2.41 (−)	2.43 (−)	2.43 (−)			
2.45	2.58 (+)	2.48 (+)	2.44 (+)	2.48	0.08	3.2
2.45	2.57 (+)	2.46 (−)	2.37 (−)			
14.5	2.64 (+)	2.47 (−)	2.48 (+)	2.49	0.08	3.4
14.5	2.42 (−)	−	2.46 (+)			
Mean	2.53	2.47	2.44	2.48	0.11	4.4
S.D.	0.13	0.12	0.05			
R.S.D. (%)	5.1	5.0	2.1			

*The sign of the deviation of the relative weight response factor from the intra-set mean is indicated in parentheses.

a means of improving sensitivity, but no decrease in the assay quantification limit was actually obtained because of increased interference from plasma components.

Linearity

Assay linearity was assessed by independently analyzing separate sets of fortified rat plasma standards and inspecting RWRFs over the standard curve concentration range. The results are presented in Table II, and representative chromatograms at concentrations spanning the standard concentration range are presented in Fig. 2. For each standard set, RWRF values were randomly distributed about the mean over the concentration range 0.012–14.5 µg/ml, with an intra-set RWRF R.S.D. of 2–5%. There was no significant difference among the means by analysis of variance, but the data failed Bartlett's test for equality of variances ($p < 0.05$) because of the low variance for set 3. The data were nevertheless pooled in order to evaluate the concentration dependence of the RWRF (Table II). The variance in the RWRF term of the pooled data was independent of concentration, and there was no significant difference between the group mean RWRF values over the concentration range 0.072–14.5 µg/ml. The mean RWRF at 0.012 µg/ml was significantly higher than the mean RWRF at other concentrations ($p < 0.05$), but the maximum difference between means was only 7.5%. Overall, the estimated RWRF R.S.D. was 4%. The assay quantification limit was defined as 12 ng/ml (1 ng injected on-column), at which the signal-to-noise ratio was 10:1 (Fig. 2).

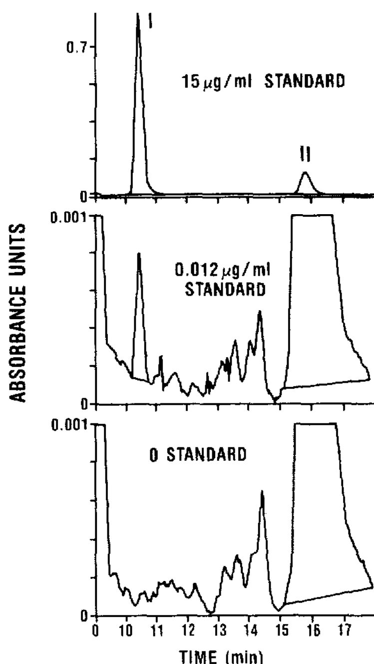

Fig. 2. Representative chromatograms of I-fortified rat plasma standards.

Precision and accuracy

The precision and accuracy of the method were established by independently assaying the same lot of QC samples in triplicate on three separate occasions. As shown in Table III, the intra-assay R.S.D. was $\leq 5\%$ at QC concentrations of 0.04, 0.49, and 7.4 μg/ml. There was significant inter-assay difference in results for the 0.04 μg/ml QC, resulting in an estimated inter-assay R.S.D. of 6%. At the higher concentrations, the estimated inter-assay R.S.D. was only 2–3%. This represents the combined random error from weighing, diluting, and pipetting the standards as well as from the extraction and chromatography steps. There was a consistently negative assay bias of -3 and -9% at the 7.4 and 0.49 μg/ml levels, respectively.

Plasma standard stability

QC samples were prepared at three levels (4.50, 0.300, and 0.0750 μg/ml) in rat plasma and stored at $-20°C$ for stability testing. The samples were assayed on the day of preparation, day 9, and day 33 using freshly prepared rat plasma standards. Assay results were plotted as a function of assay date and the data were analyzed by unweighted least-squares linear regression. The slope was not significantly different from zero at all three concentrations ($p > 0.15$). The pooled mean assay results (and R.S.D.) were 4.49 (5.0%), 0.293 (5.9%), and 0.0769 (7.0%), respectively. The absence of assay bias or slopes significantly different from zero indicate that I is stable in plasma for at least one month when stored at $-20°C$ in polypropylene tubes.

TABLE III

INTER-ASSAY AND INTRA-ASSAY PRECISION AND ACCURACY FOR RAT PLASMA SAMPLES

Actual concentration (μg/ml)		Concentration found (μg/ml)			Significant inter-assay difference ($p<0.05$)	Pooled estimate of inter-assay R.S.D. and error
		Assay 1	Assay 2	Assay 3		
7.44	Mean	7.20	7.29	7.23	NO	7.23
	S.D.	0.19	0.11	0.17		0.15
	R.S.D. (%)	2.6	1.5	2.3		2.0
	Error (%)	−3.2	− 2.0	−2.8		−2.8
	n	3	2	3		8
0.492	Mean	0.452	0.439	0.449	No	0.447
	S.D.	0.008	0.023	0.007		0.014
	R.S.D. (%)	1.8	5.3	1.6		3.2
	Error (%)	−8.1	−10.8	−8.7		−9.1
	n	3	3	3		9
0.0394	Mean	0.0362	0.0378	0.0405	Yes	0.0382
	S.D.	0.0010	0.0008	0.0016		0.0023
	R.S.D. (%)	2.9	2.1	4.0		6.0
	Error (%)	−8.1	− 4.1	2.8		−3.0
	n	3	2	3		8

Application

The utility of the assay was demonstrated by analyzing plasma samples from rats that received a 30 mg/kg i.p. dose of I. As shown in Fig. 3, the assay was sufficiently sensitive to quantify I at concentrations which were less than 10% of the maximum plasma concentration. The assay results for duplicate samples

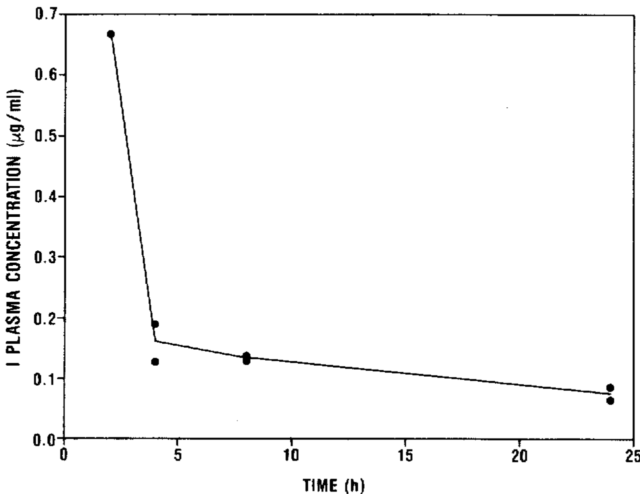

Fig. 3. Plasma concentration–time profile after a 30 mg/kg intraperitoneal dose of I in the rat.

drawn at three of the timepoints were in agreement within expected experimental error.

DISCUSSION

Under the conditions employed for both the solid-phase plasma extraction and the analytical HPLC separations, compound I should have been retained primarily by silanophilic column interactions [5]. These were shown in previous work to be more dependent on the concentration of amine modifier in the mobile phase or the pH than on the acetonitrile concentration, with the consequence that, at neutral pH or in the absence of amine modifier, a high concentration of acetonitrile could be used without causing elution of the drug. Similar results have been reported for tricyclic antidepressant drugs [8–10]. Thus, in the case of the solid-phase extraction, I in 50% acetonitrile solution was retained by the octadecylsilane columns essentially irreversibly with 100% acetonitrile or methanol as the eluting solvent. When applied to the columns in deproteinated plasma, however, the retention was still quantitative but the drug could be recovered in high yield, presumably because plasma components competed for silanol groups. Even so, the columns could be washed with up to 70% acetonitrile without causing singificant elution of the drug, and, based on the appearance of chromatograms of the extracts, most potentially interfering plasma components were eluted with the solvent wash.

The retention properties of the drug on bonded-phase silica supports were also exploited during the development of the analytical HPLC separation. An acetonitrile concentration in the mobile phase of 70% and a capacity factor of 4 were selected a priori as operating conditions. The TEA concentration was adjusted appropriately to achieve these specifications. When a plasma extract was injected and found to contain a component that coeluted with I, the peaks were resolved while keeping the capacity factor of the drug constant by a combination of TEA and acetonitrile concentration adjustments. If this approach had failed, other strategies could have been tried, including changing the sodium salt concentration or pH of the mobile phase or using a different amine modifier [5].

The limit of quantification of the assay was 12 ng/ml, which was considered adequate for animal pharmacokinetic and toxicology studies. In a preliminary investigation aimed at improving the assay sensitivity, the detector source was changed from the 254-nm mercury line to a 229-nm cadmium line. Although the latter wavelength was closer to the absorption maximum of I (234 nm), there was no improvement in the signal-to-noise ratio. More promising was the possibility of injecting a higher percentage of the plasma extract on-column. By the current procedure, 0.5 ml of plasma was extracted, and 20% of the extract was injected on-column, or an amount equivalent to 0.1 ml of plasma. In a subsequent experiment, 1.5 ml of plasma was extracted and 20% was injected on-column, indicating that the assay sensitivity could be improved at least three-fold by more efficient injection of the 0.5-ml plasma extract.

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REFERENCES

1 J.M. Braughler, J.F. Pregenzer, R.L. Chase, L.A. Duncan, E.J. Jacobsen and J.M. McCall, J. Biol. Chem., 262 (1987) 10438.
2 E.D. Hall, J. Neurosurg., in press.
3 E.D. Hall, P.A. Yonkers, J.M. McCall and J.M. Braughler, J. Neurosurg., in press.
4 E.D. Hall, P.A. Yonkers and J.M. McCall, Circ. Shock, submitted for publication.
5 J.W. Cox and R.H. Pullen, J. Chromatogr., 424 (1988) 285.
6 G.W. Snedecor and W.G. Cochran, Statistical Methods, Iowa State University Press, Ames, IA, 7th ed., 1980, p. 252.
7 E. Lundanes, J. Dohl and T. Greibrokk, J. Chromatogr. Sci., 21 (1983) 235.
8 A. Sokolowski and K.-G. Wahlund, J. Chromatogr., 189 (1980) 299.
9 J.S. Kiel, S.L. Morgan and R.K. Abramson, J. Chromatogr., 320 (1985) 313.
10 G.L. Lensmeyer and M.A. Evenson, Clin. Chem., 30 (1984) 1774.