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Simultaneous quantification of an anti-inflammatory compound (DuP 697) and a potential metabolite (X6882) in human plasma and urine by high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic (HPLC) method using fluorescence detection has been developed for the simultaneous analysis of low nanogram concentrations of an anti-inflammatory drug, 5-Bromo-2-(4-fluorophenyl)-3-[4-(methylsulfonyl)phenyl]thiophene (DuP 697), and a potential metabolite (X6882) in human plasma and of DuP 697 in human urine. This assay method used an EM Separations Lichrospher C_{18} endcapped column. The mobile phase was acetonitrile–water (75:25, v/v). The detection of DuP 697 and X6882 was by fluorescence at excitation and emission wavelengths of 256 and 419 nm, respectively. The chromatographic system could separate DuP 697 from X6882, the external standard (anthracene), and other endogenous substances present in human plasma. In human plasma the limits of quantification for DuP 697 and X6882 were 3 and 20 ng/ml, respectively; the limit of quantification for DuP 697 in human urine was 5 ng/ml. These compounds were shown to be stable in frozen (−20°C) human plasma and urine for at least 9 weeks. The assay described has been used to characterize DuP 697 pharmacokinetics after oral administration in humans.

1. Introduction

5-Bromo-2-(4-fluorophenyl)-3-[4-(methylsulfonyl)phenyl]thiophene (DuP 697, I) (Fig. 1), a cyclooxygenase inhibitor with unique pharmacological properties [1], is currently under Phase I clinical development as an anti-inflammatory drug. To support the clinical development of this compound, we developed an assay which allows simultaneous determination of I and its potential metabolite, 2-(4-fluorophenyl)-3-[4-(methylsulfonyl)phenyl]-5-(methylsulfonyl)thiophene (X6882, II), in human plasma and I in human urine. This paper describes an HPLC method utilizing fluorescence detection to simultaneously quantify low nanogram concentrations of I and II, in human plasma, and I in human urine. Compound II could not be separated from variable interference in control

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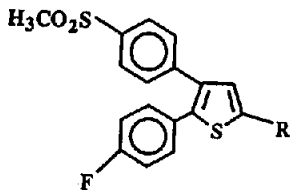

Compound	R
DuP 697	Br
X6882	SO_2CH_3

Fig. 1. Structures of DuP 697 (I) and X6882 (II).

human urine. Compound II has been identified in rats, dogs and monkeys [2].

2. Experimental

2.1. Materials

Compounds I and II were obtained from The DuPont Merck Pharmaceutical Company (Wilmington, DE, USA). Anthracene was purchased from Sigma (St. Louis, MO, USA). Acetonitrile and ethyl ether (both HPLC grade) were purchased from J.T. Baker (Phillipsburg, NJ, USA) and Baxter (McCaw Park, IL, USA), respectively. Sodium hydroxide was purchased from EM Sciences (Gibbstown, NJ, USA).

2.2. Equipment

The HPLC system consisted of a WISP 712 automatic injector (Waters Chromatography Division, Millipore, Milford, MA, USA), a Waters 600 multisolvent delivery system, a Shimadzu RF-551 fluorescence detector (Shimadzu Corporation, Colombia, MD, USA), and a PE-Nelson Series 900 Data Acquisition system with Turbochrom III software (PE-Nelson, Cupertino, CA, USA).

2.3. Chromatographic conditions

A Lichrospher 100, RP-18 endcapped column (250 × 4.6 mm I.D; 5 μm particle size) from EM Separations (Gibbstown, NJ, USA) was used for the HPLC system. The mobile phase used was acetonitrile–water (75:25, v/v) at a flow-rate of 0.8 ml/min. The excitation and emission wavelengths of the fluorescence detector were set at 256 and 419 nm, respectively. All analyses were performed at ambient temperature.

2.4. Sample preparation

Working standard solutions of I and II in acetonitrile (plasma assay only) were pipetted into 15-ml culture tubes and evaporated to dryness under nitrogen at room temperature. A 1-ml sample of human plasma (or urine) was added. After addition of 1 ml of 0.1 *M* NaOH, the plasma (or urine) was vortex-mixed and extracted with 10 ml of ethyl ether on a rotary shaker for 15 min. After centrifugation at 1800 *g* for 15 min, 7 ml of the organic layer were removed and dried under nitrogen at room temperature. The residue was reconstituted with 250 μl of acetonitrile containing 12 ng/ml of anthracene as the external standard. After 10 s vortex-mixing and 5 min sonication, 50 μl of this acetonitrile solution were injected onto the chromatographic system.

2.5. Quantification

Calibration standards in human plasma were prepared by adding known amounts of I and II to 1 ml of control plasma in order to produce final concentrations of 3–250 ng/ml (~7.3–607.8 nmol/l) and 20–800 ng/ml (~48.7 nmol/l–1.9 μmol/l), respectively. Calibration standards in human urine were prepared to produce final concentrations for I between 5–250 ng/ml (~12.2–607.8 nmol/l). The standard curves were constructed by plotting the peak-area ratios of the compounds to the external standard against the concentrations of these compounds in plasma or urine. The extracted standard curves for I and II were split at 20 and 100 ng/ml, respectively to give two linear segments of the standard curve for each compound.

3. Results and discussion

3.1. Sample preparation

Compound I and its potential metabolite are neutral compounds. Therefore, no pH change is required to extract them from plasma. However, it was observed that basification with sodium hydroxide gave cleaner chromatograms. Ethyl ether proved to be the best solvent for extraction when compared with either ethyl acetate or methylene chloride. After evaporation under nitrogen at room temperature, the residue was reconstituted with acetonitrile, vortex-mixed, and sonicated for 5 min in order to facilitate solubilization of these compounds. Without sonication, the reconstitution of the compounds was variable and incomplete.

3.2. Column selection

Several C_{18} columns were evaluated for separating I from its potential metabolite. The C_{18} endcapped column currently used gave significantly better separation of the compounds without complicated gradients or mobile phase.

3.3. External standard selection

On testing several analogs of I and not finding an acceptable one, we found anthracene which could be separated under the HPLC conditions used and detected under the fluorescence excitation and emission conditions. However, anthracene would sublime during nitrogen evaporation at room temperature, and therefore, could not be used as an internal standard. We, therefore, used it as an external standard.

3.4. Selectivity

Fig. 2 shows chromatograms of a drug free plasma and of a spiked plasma sample containing I, II, and anthracene. The two compounds were separated from anthracene and other endogen-

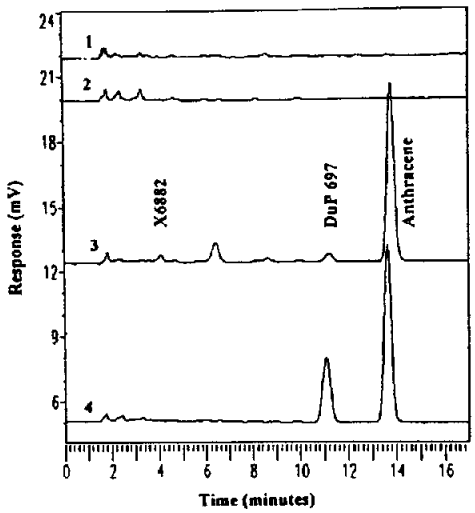

Fig. 2. Examples of chromatograms of DuP 697 (I), X6882 (II) and anthracene in human plasma. (1) Drug free human plasma; (2) dosed subject predose plasma; (3) drug free human plasma spiked with 3 and 20 ng/ml of I and II, respectively; (4) plasma sample at 4 h from subject given a single 10-mg oral dose of I (23.3 ng/ml of I). Samples processed as described in the section on sample preparation.

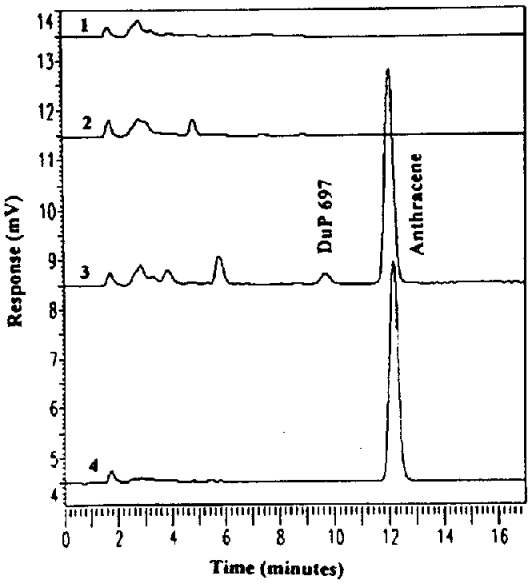

Fig. 3. Examples of chromatograms of DuP 697 (I) and anthracene in human urine. (1) Drug free human urine; (2) dosed subject predose urine; (3) drug free human urine spiked with 5 ng/ml of I; (4) 4 to 8 h urine sample from subject given a single 10-mg oral dose of I (none of the compounds were found in subject samples). Samples processed as described in the section on sample preparation.

Table 1
Typical standard curve coefficients for the HPLC assay of I and II in human plasma and I in human urine. Linear regression analysis was used to construct standard curves

Compound	Concentration range (ng/ml)	Intercept	Slope	Correlation coefficient
Human plasma				
I	3–20	−0.002743	0.019017	0.9998
	20–250	0.013811	0.018259	1.0000
II	20–100	−0.004851	0.001927	0.9993
	100–800	−0.004935	0.001856	0.9995
Human urine				
I	5–20	−0.008550	0.019132	0.9997
	20–250	−0.144439	0.018968	0.9969

ous substances present in human plasma. The retention times for II, I, and anthracene were 4.1, 11.0, and 13.6 min, respectively. The total run time was 17 min. Fig. 3 shows similar chromatograms for human urine. We could not validate the method for II in human urine since there was variable interference from control urine in the chromatographic region corresponding to the retention time for this compound.

3.5. Standard curve characteristics

Linear regression analysis was used to construct standard curves for the compounds. Standard curves were split into low and high standard

Table 2
Inter-day and intra-day precisions expressed as percent coefficient of variation for the HPLC assay from human plasma and urine

Compound	Concentration (ng/ml)	Coefficient of variation (%)		
		Intra-day[a]		Inter-day[b]
		Day 1	Day 2	
Human plasma				
I	3	3.96	11.52[c]	12.86
	20	6.91	8.42	1.85
	250	1.57	7.47	10.06
II	20	7.02	16.92	17.17
	100	6.21	4.39	3.83
	800	3.60	1.25	0.35
Human urine				
I	5	2.13	4.57	4.19
	20	1.23	1.44	2.78
	250	1.90	2.02	0.82

[a] $n = 6$.
[b] $n = 3$.
[c] $n = 5$, excluding one sample with a percent difference from spiked concentration equal to 95%. It was suspected that this was a spiking error. %C.V. was 26.60 when this sample was included in the analysis.

curves. The regression analysis showed an average correlation coefficient of 0.9974 or better for both compounds. Typical standard curve coefficients are given in Table 1.

3.6. Inter-day and intra-day precision

The intra-day assay precision was determined in the following fashion. Six (urine) to seven (plasma) sets (six samples per set) of spiked plasma or urine control samples covering the assay concentration ranges were assayed on 2 separate days. Intra-day precision results on the two validation days, expressed as percent coefficient of variation (% C.V.), are shown in Table 2. The inter-day assay precision was assessed using the calculated concentrations of one spiked control from each set obtained on each of three different days. The intra-day assay precision of all but one concentration of I in plasma on one day were within the cut-off criterion of ±20%. The inter-day assay precision at all concentrations was within the criterion.

3.7. Accuracy, robustness and reproducibility

Accuracy was evaluated using blind-coded plasma and urine samples (prepared by someone other than the analyst) assayed on a single day. Results from three of six samples (2nd to 4th) at each level are shown in Tables 3 and 4. The assay could accurately determine concentrations of I and II in plasma, within the assay validation criterion of ±20% difference in all but one unknown sample for each compound; and it could also determine I in human urine, within the assay validation criterion of ±20% difference. Figs. 4 and 5 give the measured concentrations of the lowest quality control (QC) samples during the plasma and urine assays,

Table 3
Accuracy of the HPLC assay in human plasma using blind coded samples

Nominal concentration (ng/ml)	Concentration found (ng/ml)	Percent difference[a]
Compound I		
3	3.16	5.33
	3.17	5.67
	3.01	0.33
12.5	12.45	−0.40
	12.58	0.64
	12.42	−0.64
62.5	62.83	0.53
	63.24	1.18
	62.94	0.69
250	258.90	3.56
	258.60	3.44
	257.44	2.98
Compound II		
20	20.63	3.15
	24.05	20.25
	21.51	7.55
62.5	59.60	−4.64
	61.05	−2.32
	60.64	−2.98
250	251.69	0.68
	239.51	−4.20
	245.17	−1.93
800	814.78	1.85
	809.56	1.20
	796.57	−0.43

[a] Percent difference between concentration found and nominal concentration.

Table 4
Accuracy of the HPLC assay of I in human urine using blind coded samples

Nominal concentration (ng/ml)	Concentration found (ng/ml)	Percent difference[a]
5	5.07	1.40
	5.50	10.00
	5.37	7.40
20	20.29	1.45
	19.52	−2.40
	21.75	8.75
50	58.46	16.92
	53.99	7.98
	57.14	14.28
250	251.04	0.42
	249.27	−0.29
	251.14	0.46

[a] Percent difference between concentration found and nominal concentration.

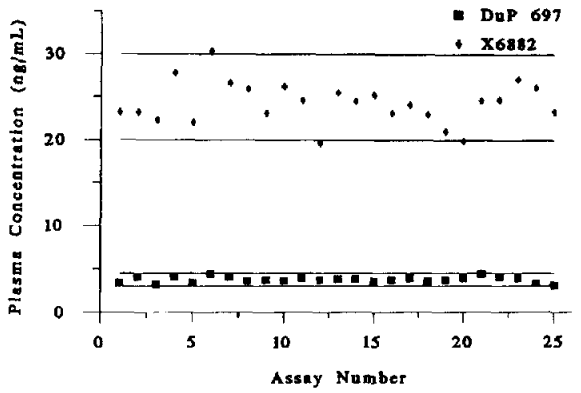

Fig. 4. Results of plasma QC samples from 25 assay runs over a period of 9 months. Concentrations shown are 3.75 and 25 ng/ml for I and II, respectively. Horizontal lines show acceptable limits (±20% of nominal concentration).

respectively. These plasma QC results are from 25 assay runs over a period of 9 months and the urine QC results are from 21 assay runs over a period of 11 months. This data shows the robustness and reproducibility of the assay.

3.8. Limit of quantitation

The limit of quantitation (LOQ) was the lowest standard concentration that could be determined with acceptable accuracy and precision. The LOQs were 3 and 20 ng/ml for I and II in human plasma, respectively and 5 ng/ml for I in human urine. The limit of detection was ca. 0.5 ng/ml.

3.9. Drug stability

The stability at −20°C of I at 20 and 100 ng/ml, and of II at 100 and 400 ng/ml in heparinized human plasma and I at 10 and 250 ng/ml in human urine was examined. Stability results are shown in Table 5. The compounds were found to be stable (<5% loss) in frozen heparinized plasma and urine for at least 9 weeks. Stock solutions in acetonitrile were also stable at 4°C for at least 21 weeks (~5 months). The compounds in plasma were also found to be stable (<9% loss) after 2 freeze–thaw cycles.

3.10. Recovery

The percent recovery of the compounds, over the entire concentration range, was determined by comparing peak areas of extracted standards to unextracted standards. The mean percent extraction efficiencies (for 6 to 7 concentrations, 6 replicates each) for I and II in human plasma

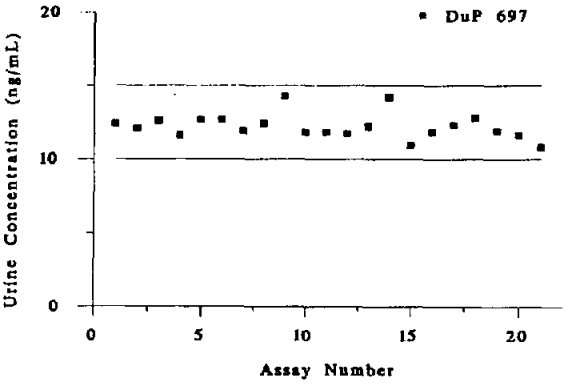

Fig. 5. Results of urine QC samples from 21 assay runs over a period of 11 months. Concentrations shown are for 12.5 ng/ml of I. Horizontal lines show acceptable limits (±20% of nominal concentration).

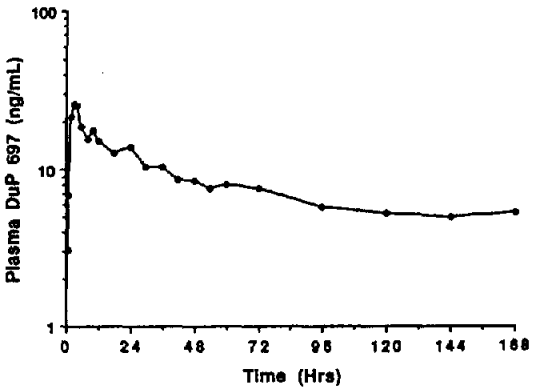

Fig. 6. Plasma concentration–time profile of I following administration of a single oral dose of 10 mg to a healthy subject.

Table 5
Stability of I and II in acetonitrile at 4°C, heparinized human plasma at −20°C, and human urine at −20°C; and human plasma after two freeze–thaw cycles

Test	Concentration (ng/ml)[a]					
	Acetonitrile		Human plasma		Human urine	
	20 ng/ml	100 ng/ml	20 ng/ml	100 ng/ml	10 ng/ml	250 ng/ml
Compound I						
0 weeks	19.51 (100.00)[b]	101.03 (100.00)	19.18 (100.00)	93.35 (100.00)	9.14 (100.00)	255.53 (100.00)
9 weeks			22.22 (115.85)	94.99 (101.76)	8.69 (95.02)	254.65 (99.62)
21 weeks	21.4 (109.69)	103.02 (101.97)				
Freeze–thaw stability						
One cycle			19.33 (100.00)[c]	89.72 (100.00)		
Two cycles			18.15 (93.90)	92.62 (103.23)		
	Acetonitrile		Human plasma			
	100 ng/ml	400 ng/ml	100 ng/ml	400 ng/ml		
Compound II						
0 weeks	93.59 (100.00)[b]	398.25 (100.00)	91.90 (100.00)	390.27 (100.00)		
9 weeks			96.09 (104.59)	401.42 (102.59)		
21 weeks	107.72 (115.10)	430.25 (108.04)				
Freeze–thaw stability						
One cycle			106.01 (100.00)[c]	414.95 (100.00)		
Two cycles			104.89 (98.94)	379.61 (91.48)		

[a] All values are expressed as mean of 2 samples.
[b] Percent remaining from 0 week mean value.
[c] Percent remaining after the first freeze–thaw cycle.

were 95.0 ± 17.5 and 112.0 ± 11.5 respectively, and for I in human urine was 102.8 ± 5.1.

3.11. Analysis of clinical samples

The method described has been successfully applied to the quantification of I in plasma and urine samples from the first study in healthy volunteers. The time course of the concentration of I in plasma from a representative subject given a 10-mg oral dose of I is shown in Fig. 6. Compound II was not detectable in subject

plasma samples. Compound I was not detectable in subject urine samples.

4. Conclusions

An HPLC method is reported for the determination of I and its potential metabolite in human plasma and for I in human urine. The method is specific, sensitive, and robust. The method has been successfully applied to the analysis of plasma and urine samples from pharmacokinetic studies of I in humans.

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