Pergamon

Journal of Pharmaceutical & Biomedical Analysis, Vol. 12, No. 9, pp. 1163–1172, 1994

0731–7085/94 $7.00 + 0.00

0731–7085(94)E0041–X

Determination of DuP 128, an ACAT inhibitor and its sulphoxide and sulphone metabolites in human plasma by liquid chromatography

CHII-MING LAI,* BERNICE BROGDON, CHECK Y. QUON and HENRY J. PIENIASZEK JR

Drug Metabolism and Pharmacokinetics Section, The DuPont Merck Pharmaceutical Company, Stine-Haskell Research Center, Building 112, Elkton Road, Newark, DE 19714, USA

Abstract: A sensitive and specific high-performance liquid chromatographic (HPLC) method with fluorescence detection was developed for the simultaneous determination of DuP 128 (N'-(2,4-difluorophenyl)-N-[5-(4,5-diphenyl-1H-imidazol-2-ylthio)pentyl]-N-hepthylurea), an ACAT inhibitor, its sulphone metabolite (XB277), and the separate determination of sulphoxide metabolite (XC164) in human plasma. After deproteinizing plasma samples with acetonitrile, the organic layer, created by adding approximately 0.25 g of NaCl, was removed, evaporated to dryness, and the residue then reconstituted with 400 μl of acetonitrile. The acetonitrile layer was washed with 5 ml of hexane and then 50 μl was injected into the HPLC. DuP 128 and XB277 were simultaneously quantified using a YMC basic column and fluorescence detection (λ_{Ex} = 270 nm and λ_{Em} = 385 nm). XC164 was quantified using a Waters μBondpack C_{18} reversed-phase column and fluorescence detection (λ_{Ex} = 270 nm and λ_{Em} = 365 nm). The relationship between the peak height and plasma concentrations best fit a power curve and showed an average correlation coefficient of >0.99 over a concentration range of 1–200 ng ml^{-1} for DuP 128 and XC164 and 2.5–200 ng ml^{-1} for XB277. Good intraday and interday assay precisions (RSD <10%) and accuracy (<14%) for all three compounds were observed. The methods were sufficiently sensitive and selective to quantify plasma concentrations of DuP 128 and its sulphoxide and sulphone metabolites after oral administration of single or multiple dose(s) of >350 mg of DuP 128 to healthy subjects.

Keywords: *DuP 128; plasma; reversed-phase liquid chromatography; fluorescence detection; quantitative determination; ACAT inhibitor; sulphoxide and sulphone metabolites.*

Introduction

DuP 128, N'-(2,4-difluorophenyl)-N-[5-(4,5-diphenyl-1H-imidazole-2-ylthio)pentyl]-N-heptylurea (Fig. 1), is a potent acyl-CoA: cholesterolacyltransferase [ACAT] inhibitor both *in vitro* and *in vivo* in animals [1–4]. The compound is being developed by The DuPont Merck Pharmaceutical Company as a hypocholesterolemic agent and is currently undergoing clinical evaluation. To facilitate analysis of plasma samples from these clinical studies, a sensitive and selective LC method was developed and validated for the quantification of DuP 128 and two of its metabolites in human plasma. The assay described herein has been successfully applied to clinical study samples.

Experimental

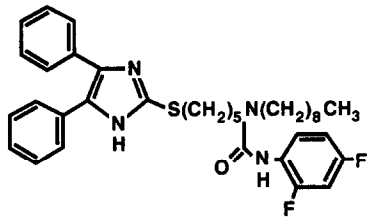
Chemicals and reagents

DuP 128 (N'-(2,4-difluorophenyl)-N-[5-(4,5-diphenyl-1H-imidazol-2-ylthio)pentyl]-N-heptylurea), XC164 (N'-(2,4-difluorophenyl)-N-((5-(((4,5-diphenyl-1H-imidazol-2-yl)sulphinyl))phenyl))-N-heptylurea), and XB277 (N'-(2,4-difluorophenyl)-N-((5-(((4,5-diphenyl-1H-imidazol-2-yl)sulphonyl))pentyl))-N-heptylurea) were synthesized at The DuPont Merck Pharmaceutical Company (Wilmington, DE). Unless otherwise stated, all solvents and reagents used were HPLC grade. Acetonitrile and hexane were purchased from J.T. Baker Inc. (Phillipsburg, NJ). Sodium chloride was supplied by VWR Scientific (Bridgeport, NJ). Frozen heparinized control human plasma was supplied by Biological Specialities Co. (Lansdale, PA).

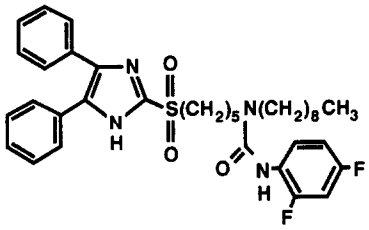
Standard solutions

Primary standard solutions of DuP 128, XC164 and XB277 were prepared in acetonitrile at nominal concentrations of 20 μg ml^{-1} (free base). Working standard solutions con-

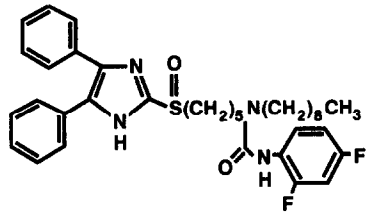
*Author to whom correspondence should be addressed.



DuP 128



XB 277



XC 164

Figure 1
Chemical structure of (A) DuP 128, and its two human metabolites (B) XC164 and (C) XB277.

taining a mixture of DuP 128, XC164 and XB277 were used for the preparation of plasma standards. These were prepared by diluting equal aliquots from each stock solutions with acetonitrile to yield nominal concentrations of 4, 3, 2, 1.5, 1, 0.5, 0.2, 0.1, 0.05 and 0.02 $\mu g\ ml^{-1}$ of each compound. All primary and working standard solutions were prepared monthly and kept at 4°C and showed no degradation over that time period. Fifty µl of each working standard was added to 1 ml of blank plasma to yield final nominal concentrations (of each compound) of 200, 150, 100, 75, 50, 25, 10, 5, 2.5 and 1 $ng\ ml^{-1}$ in plasma.

Instrumentation and chromatographic conditions

The chromatographic system was comprised of a Waters 590 pump, 710A WISP sample injector (Waters Associates, Milford, MA), and a Shimadzu model RF-551 fluorescence HPLC monitor (Shimadzu Scientific Instruments, Inc., Columbia, MD). Chromatography was conducted at 40°C using a SYS-TEC CH-1448 Dual Zone Temperature Controller (Systec, Inc., Minneapolis, MN). Separation was achieved using a 4.6 mm × 25 cm, 5 µm YMC basic Column (YMC, Inc., Wilmington, NC) for DuP 128 and XB277 (System 1) and using a Waters 4.6 mm × 25 cm, 10 µm µBondapak C_{18} column for XC164 (System 2). The mobile phase was acetonitrile–0.005 M phosphoric acid (65:35, v/v) for System 1 and acetonitrile–0.1 M phosphoric acid (70:30, v/v) for System 2. The flow rates of the mobile phase were 1.4 and 1.2 $ml\ min^{-1}$ for System 1 and System 2, respectively. The detector was set at an excitation wavelength of 270 nm for both systems and the emitted fluorescence was monitored at 385 nm for both DuP 128 and XB277 (System 1) and 365 nm for XC164 (System 2). Peak heights for the drug and metabolites were measured using a HP 3365 ChemStation (Hewlett–Packard, Avondale, PA).

Sample preparation

To 2.0 ml of plasma, 6 ml of acetonitrile was added to denature the plasma proteins. The sample was vortexed for 5 s and centrifuged (Sorval® RT 6000D, DuPont Company, Wilmington, DE) at 3000 rpm for 5 min at room temperature. The aqueous layer was carefully decanted into a 6 × 125 mm culture tube containing approximately 0.25 g of NaCl. The acetonitrile was separated from plasma water by vortexing for 30 s, mechanical shaking (Eberback Co., Ann Arbor, MI) at high speed for 15 min, and then centrifuging for 10 min at 3000 rpm. The top acetonitrile layer was transferred to another clean culture tube (6 × 125 mm) and evaporated to dryness under a

nitrogen stream at ambient temperature. Four hundred μl of acetonitrile and 5 ml of hexane were added to dissolve the residues. After shaking the sample at high speed for 5 min and then centrifuging for 10 min at 3000 rpm, the hexane layer containing endogenous compounds from plasma was discarded. Two 150-μl aliquots of the acetonitrile layer were transferred to glass WISP® vials and 50 μl was injected and analysed by HPLC Systems 1 and 2.

Validation procedures

Plasma standards ranging from 1 to 200 ng ml^{-1} each of mixtures of DuP 128, XC164 and XB277 were prepared and analysed on the same day. The intraday precision of the assay was assessed using raw peak heights and obtaining Relative Standard Deviations (RSD) for each of the three compounds (six replicates) at concentrations of 1, 10, 75 and 200 ng ml^{-1}.

Similarly, plasma samples containing mixtures of DuP 128, XC164 and XB277 at 1, 10, 75 and 200 ng ml^{-1} each were prepared daily and analysed on three different days. The interday precision of the method was again assessed as RSD. The interday precision was also evaluated by determining the variability of the regression coefficients for best fit of standard curves prepared on three different days. The specificity of the assay was checked by assaying pre-dose plasma samples from several different subjects and ensuring that no endogenous interferences were observed. The accuracy of the method was assessed over the concentration range of 1–200 ng ml^{-1} for DuP 128 and its metabolites. The accuracy of the method was assessed as absolute value of % difference between the assayed concentration and spiked concentration at 1.25, 3.13, 12.5, 62.5, 125.0 and 187.5 ng ml^{-1} of all three compounds in the same sample:

$$\% \text{ difference} = \frac{(\text{found conc.} - \text{spiked conc.})}{\text{spiked conc.}} \times 100\%. \quad (1)$$

Preliminary human study

After giving written informed consent, a group of 48 healthy male volunteers received 350 mg–15 g of DuP 128 orally. Blood samples were drawn in heparinized tubes at 0, 1, 2, 3, 4, 6, 8, 12, 16 and 24 h following drug adminstration. The blood samples were centrifuged, and the plasma was separated then stored at −20°C until analysed. Plasma DuP 128, XC164 and XB277 concentration–time data were analysed using standard noncompartmental methods for determination of terminal half-lives.

Results

Detector and chromatography optimization

Prior to LC analysis, the fluorescence excitation and emission wavelengths were optimized as follows. Single component solutions (in acetonitrile as a solvent) were loaded in the 12 μm quartz flow cell of the fluorescence detector. The excitation wavelength was scanned between 200 and 450 nm. Maximum response was observed at an excitation wavelength at 270 nm for all three compounds. Setting the excitation wavelength at 270 nm, the emission spectrum was scanned from 300 to 450 nm. Maximum responses were observed at 405, 350 and 370 nm, respectively, for DuP 128, XC164 and XB277.

The effect of phosphoric acid concentration in the LC mobile phase on separation of the three compounds is shown in Fig. 2. The phosphoric acid to acetonitrile phase volume ratios was in all cases 65:35. At phosphoric acid concentrations between 0.001 and 0.1 M, the retention times were nearly constant for XC164 (12.37–12.94 min), and XB277 (16.08–16.50 min). Elution of DuP 128 from YMC basic column was very sensitive to the phosphoric acid concentration in the mobile phase. At low phosphoric acid concentrations, DuP 128 elution was inhibited and the retention time lengthened significantly (from 9.37 to 33.90 min as the phosphoric acid concentration was decreased from 0.1 to 0.001 M). Co-elution of DuP 128 with either XC164 or XB277 could be prevented when the acid concentration was reduced to <0.01 M. Thus 0.005 M phosphoric acid was selected for the mobile phase to obtain the best combination of the following: column selectivity, short, reproducible retention times, high mass sensitivity, and prolonged column life.

Chromatography and assay validation

Chromatography using the YMC basic and μBondapak C_{18} columns allowed the resolution of DuP 128, XC164 and XB277 from endogenous plasma components. Represen-

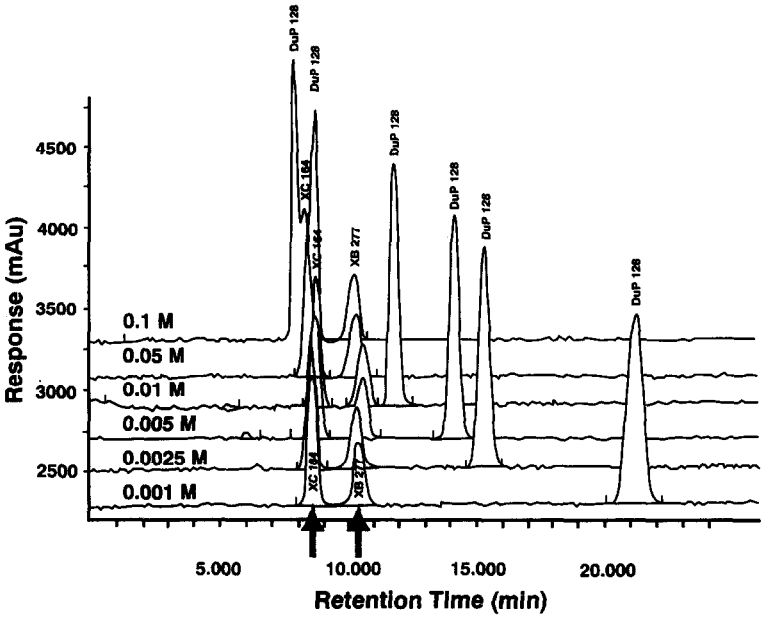


Figure 2
Influence of the phosphoric acid concentration on the elution of DuP 128, and its sulphoxide and sulphone metabolites from HPLC with a YMC basic column.

Table 1
Intraday assay precision for DuP 128, XC164 and XB277 in human plasma

	Peak height response (mAu)			
Determination	1 or 2.5 ng ml^{-1}*	10 ng ml^{-1}	75 ng ml^{-1}	200 ng ml^{-1}
DuP 128				
Mean (n = 6)	56.62	528.19	4200.38	11962.53
SD	1.84	8.49	90.60	398.04
RSD (%)	3.24	1.61	2.16	3.33
XC164				
Mean (n = 6)	227.29	2005.03	16518.09	46362.86
SD	12.66	37.77	549.69	1482.99
RSD (%)	5.57	1.88	3.33	3.20
XB277				
Mean (n = 6)	55.08	213.17	1567.10	4192.61
SD	2.38	5.22	51.93	354.49
RSD (%)	4.32	2.45	3.31	8.45

*Actual concentration added: 1 ng ml^{-1} for both DuP 128 and XC164 and 2.5 ng ml^{-1} for XB227.

tative chromatograms of control plasma and control plasma spiked with 50 ng ml^{-1} of each of three compounds and a 12-h post-dose plasma sample from one of eight subjects after oral dosing with 350 mg of DuP 128 are shown in Figs 3 and 4, respectively. Analysis of control plamsa indicated the absence of interfering endogenous compounds, confirming adequate assay specificity. Retention times for DuP 128, XC164, and XB277 were 11.60, 8.29 and 10.31 min for YMC basic column, respectively, and were 7.07, 10.18 and 12.43 min for C_{18} column, respectively.

The intraday reproducibility of the assay were determined by the analysis of six replicate plasma spikes at four different plasma concentration levels ranging from 1 (for DuP 128 and XC164) or 2.5 (for XB277) to 200 ng ml^{-1} for all three compounds (Table 1). The precision showed a maximum RSD of peak height response of <4% for DuP 128, <6% for XC164 and <9% for XB277. The quantification limit based on intraday precision, therefore, was 1 ng ml^{-1} for DuP 128 and XC164 and 2.5 ng ml^{-1} for XB277.

The interday precision results are shown in

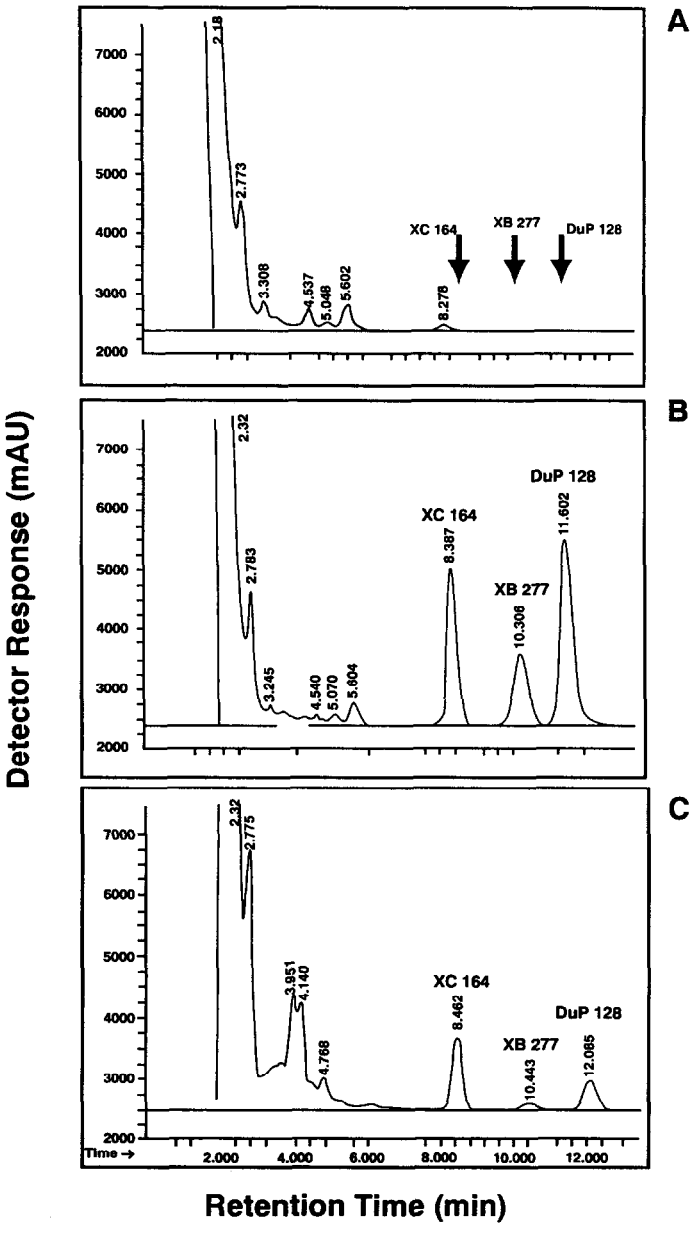


Figure 3
Representative chromatograms (YMC basic column) of DuP 128, XC164 and XB277 in human plasma. (A) Control plasma, (B) control plasma spiked with 50 ng ml^{-1} of each of DuP 128, XC164 and XB277 and (C) post-dose plasma sample from human subject 12 h after oral dosing with 350 mg of DuP 128; the concentrations of DuP 128, XC164 and XB277 are equivalent to 8.32, 18.24 and 3.62 ng ml^{-1}, respectively.

Table 2. The values for precision (RSD) analysed on three different days were all ≤16% over the concentration range of 1–200 ng ml^{-1}. The coefficients of variation were similar or only slightly higher than those reported for the intraday data.

The coefficients of regression of power curve fitting ($Y = aC^b$, where Y is peak height, C is drug concentration, and 'a' and 'b' are regression coefficients) from three sets of calibration curves for each of three compounds which were used in the studies (one set/day) are shown in Fig. 5. The regression analysis showed an average correlation coefficient of 0.999 for all compounds. The variation (RSD) of the regression coefficient 'a' were 10.80, 8.45 and 2.23% for DuP 128, XC164 and XB277 regressions, respectively. The RSD for the regression coefficient 'b' was extremely small for all three assays (<2%).

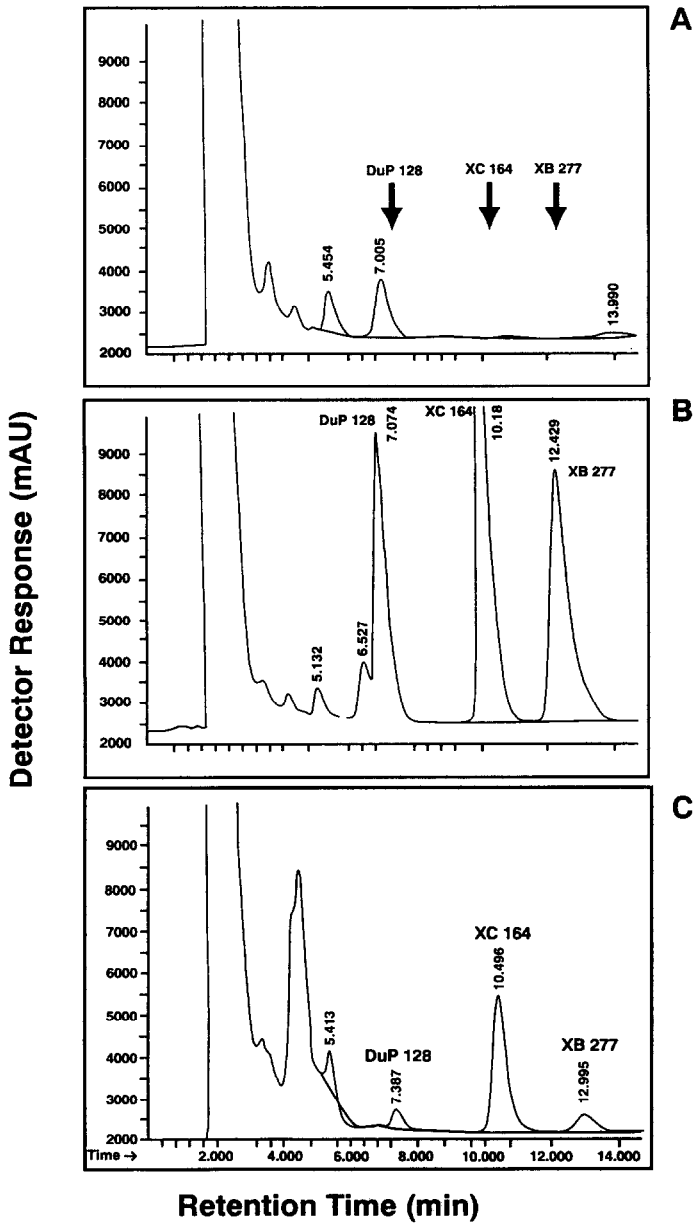


Figure 4
Representative chromatograms (μBondapak C_{18} column) of DuP 128, XC164 and XB277 in human plasma. (A) Control plasma, (B) control plasma spiked with 50 ng ml^{-1} of each of DuP 128, XC164 and XB277 and (C) post-dose plasma sample from human subject 12 h after oral dosing with 350 mg of DuP 128; the concentrations of DuP 128, XC164 and XB277 are equivalent to 8.32, 18.24 and 3.62 ng ml^{-1}, respectively.

The accuracy of the assay for the three compounds was evaluated at concentrations of 1.25, 3.13, 12.5, 62.5, 125.0 and 187.5 ng ml^{-1} of plasma. The results are presented in Table 3. The percentage difference between the spiked and measured amounts (analytical bias) was <13% for DuP 128, <9% for XC164 and <9% for XB277.

Discussion

An acid concentration-dependent elution volume of DuP 128 was observed for DuP 128, but not for either of its two metabolites. The retention time of the DuP 128 was lengthened considerably at low acid concentrations in the mobile phase. This phenomenon was probably

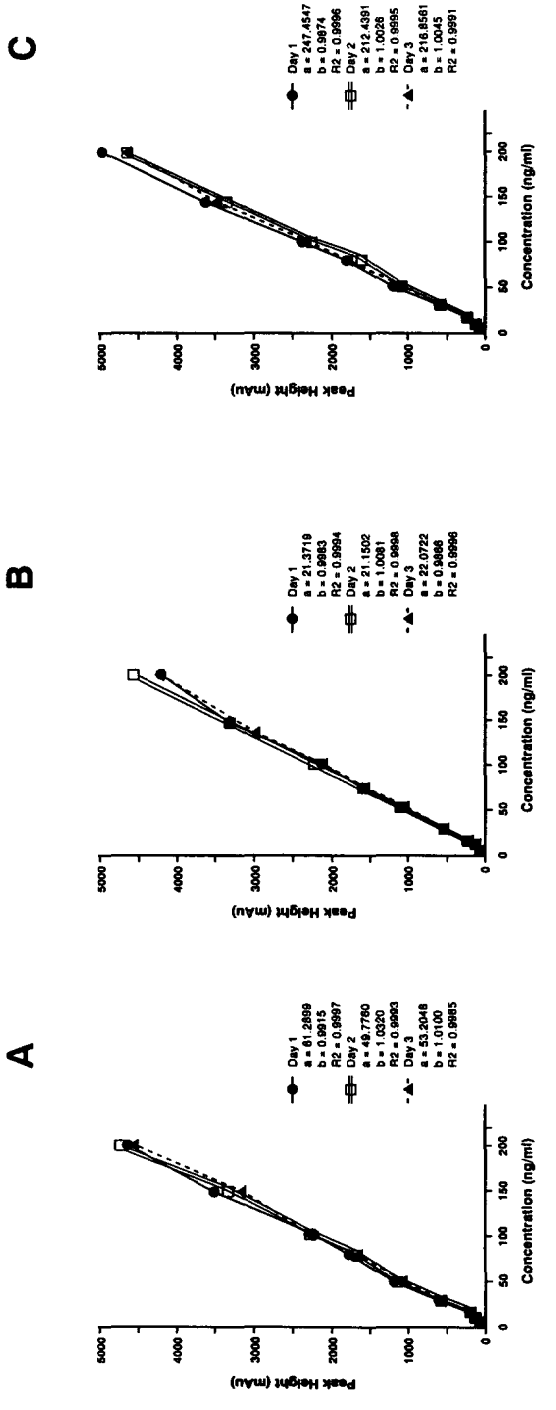


Figure 5
Calibration curves for (A) DuP 128, (B) XC164 and (C) XB277 in human plasma.

Table 2
Inter-day assay precision for DuP 128, XC164 and XB277 in human plasma

Determination	Relative standard deviation (%)			
	1 or 2.5 ng ml^{-1}*	10 ng ml^{-1}	75 ng ml^{-1}	200 ng ml^{-1}
DuP 128				
Day 1†	1.01	3.50	1.57	2.13
Day 2†	16.24	4.44	2.89	1.77
Day 3‡	3.18	1.57	2.14	3.29
XC164				
Day 1†	8.82	5.22	4.71	2.07
Day 2†	5.05	3.24	3.24	2.95
Day 3‡	5.62	1.86	3.31	3.18
XB277				
Day 1†	5.26	9.47	3.22	7.53
Day 2†	7.72	2.47	5.21	1.40
Day 3‡	4.32	2.49	3.36	8.57

*Actual concentration added: 1 ng ml^{-1} for both DuP 128 and XC164 and 2.5 ng ml^{-1} for XB227.
†$n = 3$.
‡$n = 6$.

Table 3
Accuracy data for DuP 128, XC164 and XB277 in human plasma

Determination	Concentration (ng ml^{-1})		
	Spiked	Found	% Difference*
DuP128			
A	1.25	1.38	10.11
B	3.13	3.43	9.73
C	187.50	185.66	−0.98
D	12.50	13.14	5.12
E	125.00	123.33	−1.34
F	62.50	60.95	−2.48
G	3.13	3.53	12.93
H	1.25	1.40	11.59
XC164			
A	1.25	1.27	1.52
B	3.13	2.92	−6.65
C	187.50	180.82	−3.56
D	12.50	11.48	−8.15
E	125.00	120.15	−3.88
F	62.50	57.50	−8.00
G	3.13	2.94	−5.84
H	1.25	1.25	0.18
XB277			
A	1.25	bql†	—
B	3.13	3.15	0.81
C	187.50	181.45	−3.23
D	12.50	12.15	−2.80
E	125.00	114.02	−8.79
F	62.50	59.28	−5.15
G	3.13	3.22	2.88
H	1.25	bql	—

* % Difference $= \frac{(\text{found conc.} - \text{spiked conc.})}{\text{spiked conc.}} \times 100\%$.
†bql = below the quantifiable limit.

due to higher acid molarity changing the charge density (OH^-) on the particle surface of colloid silica, and thereby increasing hydrogen bonding of the polar substituents on the DuP 128 molecule to the silica surface.

XB277, the sulphonyl analogue of DuP 128, was initially chosen to be an internal standard for this assay of clinical plasma samples. This candidate was chosen because of its structural similarities to DuP 128 and XC164 and the fact that in our preclinical studies, it was not found to be a metabolite of DuP 128 in rats or dogs [5]. The use of XB277 as an internal standard showed excellent accuracy and reproducibility; however, it was later discovered that the compound was one of the human metabolites found in subjects receiving multiple oral doses of DuP 128. Therefore, in the present assay validation, absolute peak heights were used for quantification. It should be noted that using the absolute peak height method still provided excellent precision and accuracy results for all three compounds.

DuP 128, XC164, XB277 and endogenous plasma compounds could be successfully separated on either a YMC basic column or C_{18} column. However, after repeated use (approximately 50 injections) of the C_{18} column, XC164 and XB277 were found to co-chromatograph with endogenous plasma components from some subjects. Similarly, DuP 128, after repeated use of the YMC column, was found to co-chromatograph with endogenous plasma components. Since these conditions severely limited the ruggedness of the method, it was decided not to use only one column to simultaneously separate all three compounds.

The optimized excitation and emission wavelengths were 270 and 405 nm, respectively, for DuP 128; 270 and 365 nm, respectively, for XC164; and 270 and 379 nm, respectively, for

XB277. However, at the emission wavelength of 405 nm, the intensity of emitted fluorescence for XB277 was approximately one-fifth that of its optimum condition (λ_{Em} = 370 nm). Therefore, it was not possible to quantify XB277 at the low ng ml^{-1} concentration level at λ_{Em} = 405 nm. We, therefore, used 270 nm as the excitation wavelength and 385 nm as the emission wavelength for the simultaneous determination of DuP 128 and XB277. We compromised on the quantification limit of DuP 128, but were then able to quantify XB277 simultaneously at 2.5 ng ml^{-1}. The lowest quantifiable concentration for DuP 128 with these settings was 1 ng ml^{-1}. Quantification limits were established based upon both signal intensity and variability. The lowest quantifiable concentration of the compounds was defined based on intra- and inter-day precision estimates of less than 10% RSD at that particular concentration. Additional, accuracy near the lowest quantifiable concentration needed to be less than ±15% difference from the added unknown concentration.

The present method provided the desired sensitivity for the determination of DuP 128 and its two metabolites in human plasma following the oral administration of 350 mg to 15 g DuP 128. A representative plasma concentration versus time curve from one subject receiving 350 mg and the other receiving 15 g of DuP 128 are shown in Figs 6 and 7, respectively, as an illustration of the applicability of this assay. The peak times for DuP 128, XC164 and XB277 were 6, 4 and 6 h, respectively. The maximum plasma concentration values (C_{max}) and their terminal half-lives ($t_{½}$) of DuP 128, XC164 and XB277 after 15 g oral DuP 128 were 76.5, 216.0 and 35.3 ng ml^{-1}, respectively, for C_{max} and 21.6, 14.4 and 34.4 h, respectively, for $t_{½}$. The reported $t_{½}$ values may underestimate the true terminal $t_{½}$ values since we did not collect and assay for a sufficiently long (i.e. $\geq 3t_{½}$) period of time post-drug administration. No XB277 was detected following the 350 mg oral dose of DuP 128 indicating that formation of XB277 was lower than those of XC164. Further studies are in progress to evaluate individual variability in the pharmacokinetic properties of DuP 128 and its metabolites.

In summary, a specific, sensitive and precise LC method with fluorescence detection for quantification of DuP 128 and two of its metabolites in human plasma was developed.

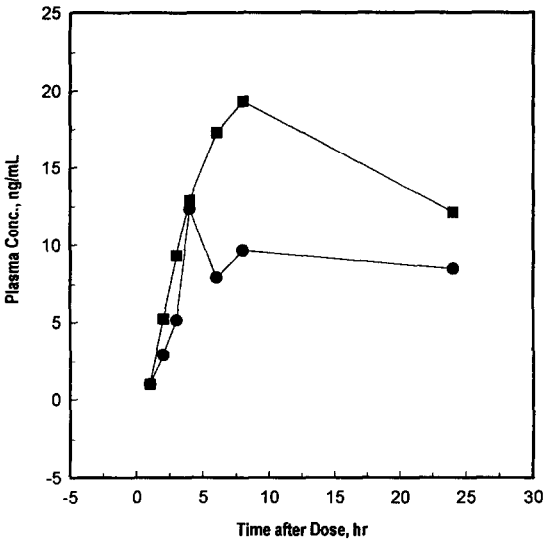


Figure 6
Time course of DuP 128 (●), XC164 (■) and XB277 (▲) in plasma after oral administration of DuP 128 at a dose of 350 mg in a subject.

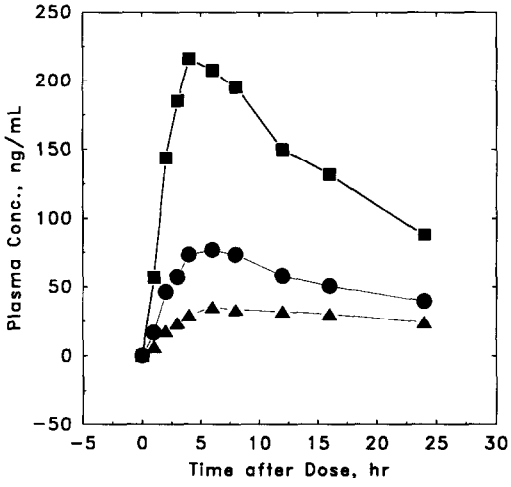


Figure 7
Time course of DuP 128 (●), XC164 (■) and XB277 (▲) in plasma after oral administration of DuP 128 at a dose of 15 g in a subject.

The assay described is capable of quantifying ≥1 ng ml^{-1} of plasma for DuP 128 and XC164 and ≥2.5 ng ml^{-1} of XB277 with an acceptable range of accuracy and intra- and inter-day variability.

References

[1] J.G. Heider, C.E. Pickers and L.A. Kelley, *J. Lipid Res.* **24**, 1127–1134 (1983).
[2] J.T. Billheimer, D.A. Crowley, C.A. Higley, R.R. Wexler, C.S. Robinson and P.J. Gillies. Ninth International Symposium on Atherosclerosis, Rosemont, IL. Abstract, p. 184 (1991).

[3] P.J. Gillies, C.S. Robinson, C.A. Higley, R.R. Wexler and J.T. Billheimer. Ninth International Symposium on Atherosclerosis, Rosemont, IL, Abstract, p. 183 (1991).
[4] J.T. Billheimer, R.R. Wexler, P.J. Gillies and L.L. Rudel. XI International Symposium on Drugs Affecting Lipid Metabolism, Montreal, Canada. Abstract p. 79 (1992).
[5] Y.N. Wong, C.A. Higley, J.M. Read, K.F. Blom, C.-M. Lai and C.Y. Quon, *Pharm. Res.* **8**(Suppl.), 321 (1991).