Determination of antifilarial compound UMF-078 and its metabolites in plasma by high-performance liquid chromatography[1]

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Abstract

UMF-078, methyl ($\pm$)-[5-(α-amino-4-fluorobenzyl)benzimidazol-2-yl]carbamate, is a new antifilarial compound being developed by the World Health Organization. In the present study, a HPLC method for the simultaneous estimation of UMF-078 and its metabolites (flubendazole, decarbamoylated flubendazole, UMF060 and decarbamoylated UMF-060) in plasma was developed, validated and applied to pharmacokinetic studies. Linearity was observed between 20 and 1000 ng/ml for decarbamoylated UMF-060 and between 10 and 500 ng/ml for other analytes. Recoveries were consistent over the concentration ranges studied for all the analytes. Variations in intra- and inter-batch accuracy and precision were within acceptable limits of $\pm$20% at the lowest limit of quantitation, whereas at higher concentrations it was $\pm$15%. The analytes showed stability up to two freeze–thaw cycles in plasma. No degradation was observed for any of the analytes even after 72 h of storing the dry plasma extracts at $-30°$C. The assay method was employed to study the pharmacokinetics of hydrochloride salt of UMF-078 in rats. The parent compound and its metabolites viz: decarbamoylated UMF-060, UMF-060 and flubendazole were quantitated in serum and the compounds could be monitored up to 168 h post-dose. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: UMF-078; Methyl ($\pm$)-[5-(α-amino-4-fluorobenzyl)benzimidazol-2-yl]carbamate; Flubendazole

1. Introduction

UMF-078, methyl ($\pm$)-[5-(α-amino-4-fluoro-benzyl)benzimidazol-2-yl]carbamate, is a new broad spectrum antifilarial agent, which is being developed by the World Health Organization (WHO). To generate reliable pharmacokinetic data for further development of UMF-078 (I, Fig. 1), a sensitive and robust assay for I and its metabolites is required.

UMF-060 (methyl ($\pm$)-[5-[(4-fluorophenyl)hydroxymethyl]-1H-benzimidazol-2-yl]carbamate), decarbamoylated UMF-060 ([2-amino-5-(α-hydroxy-4-fluorobenzyl)]benzimidazole), flubendazole (methyl ($\pm$)-[5-(4-fluorobenzoyl)-1H-benzimidazol-2-yl]carbamate), decarbamoylated flubendazole ([(2-amino-1H-benzimidazol-5-yl)-4-fluorophenyl]methanone) and decarbamoylated UMF-078 (2-amino-5-(α-amino-4-fluorobenzyl)benzimidazole) (Fig. 1) have been identified as the metabolites of I, after conducting in vitro metabolic studies of I using rat liver microsomes (unpublished report, WHO dossier).

A high-performance liquid chromatography

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UMF-078 (I)

DECARBAMOYLATED-UMF-060 (II)

UMF-060 (IV)

DECARBAMOYLATED-FLUBENDAZOLE (III)

FLUBENDAZOLE (V)

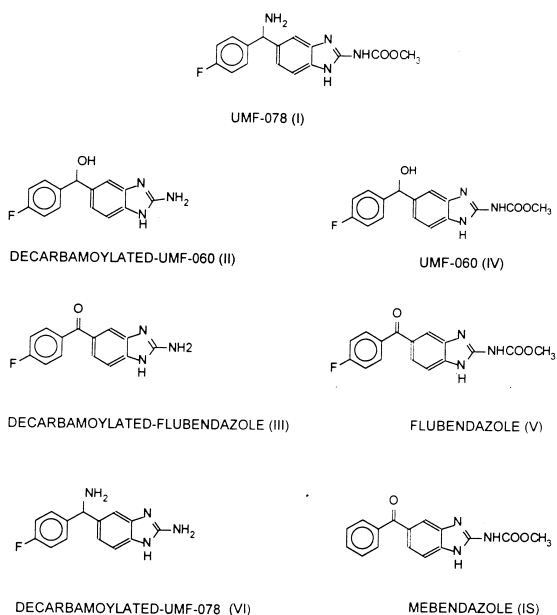DECARBAMOYLATED-UMF-078 (VI)

MEBENDAZOLE (IS)

Fig. 1. Structural formulae for the analytes and internal standard.

(HPLC) method for the simultaneous estimation of I, IV and V in monkey blood has been reported [1], which utilizes extraction of blood with diethyl ether at pH 10.5 followed by gradient elution on a HPLC system. Another method has been developed for the estimation of decarbamoylated metabolites of I viz., decarbamoylated UMF-078, IV and V, which involves extraction with ethyl acetate (unpublished report, WHO). Thus, the same sample has to be analyzed twice for the quantitation of I and its metabolites. Therefore, a method for the simultaneous estimation of I and its metabolites is needed. This communication describes the development and validation of a sensitive, accurate and reproducible HPLC assay method for the simultaneous quantitation of I and its metabolites, except DUMF-078, in plasma using mebendazole as an internal standard (I.S.) by a simple extraction technique. Accuracy, precision, effect of freeze–thaw (f–t) cycles and storage of extracted residues on the stability of I and its metabolites were used as the parameters of validation. The method was applied to study the pharmacokinetics of I by orally administering UMF-289 (hydrochloride salt of I) equivalent to 100 mg/kg of free base to male Sprague–Dawley rats.

2. Experimental

2.1. Reagents and standards

Pure reference standards (I–V) (assay>99%) were obtained from the WHO and mebendazole (used as I.S.) was a gift from Cipla India. Acetonitrile (S.D. Fine Chemicals, Boisar, India) and methanol [E. Merck (India), Mumbai, India] were of HPLC grade and used without further purification. Dimethyl sulfoxide (DMSO) of scintillation grade was purchased from Spectrochem (Bombay, India). Diethyl ether (T.K. Chemicals Enterprises, T.N., India) was purified and distilled before use. All other reagents were of analytical grade and were used without further purification. Normal human plasma (NHP) was purchased from a local blood bank. It was pooled, stored at $-30°C$ and was used within seven days.

2.2. Apparatus and chromatographic conditions

A binary HPLC pump (LC-10AD with CBM-10A communication bus station, Shimadzu, Japan) was used to pump the mobile phase [pump A: phosphate buffer (50 mM, pH adjusted to 4.0 with orthophosphoric acid) and pump B: 25% acetonitrile in methanol] at a flow-rate of 1 ml/min. Chromatographic separations were performed on a C_{18} reversed-phase column (Spheri-5, 5 μm, 100×4.6 mm I.D.) preceded by a guard column packed with the same material as in the analytical column (30×4.6 mm I.D.) (Pierce, Rockford, IL, USA). Optimum separation of I, its metabolites and the I.S. from the endogenous plasma components was achieved using gradient elution. Gradient elution started with pump A and pump B supplying 70% and 30% of the mobile phase components, respectively. The concentration of the mobile phase component in pump B increased linearly to 55% within 10.25 min and kept constant up to 13.5 min followed by a linear decrease to 30% by 17.5 min. Mobile phase solvents were filtered and degassed before use. A Model 7725i syringe loading sample injector (Rheodyne, Cotati, CA, USA) with a fixed 20-μl sample loop was used to inject the samples onto the HPLC system. After elution the compounds were monitored using a Model SPD-10AV, UV–Vis detector

(Shimadzu) set at 291 nm. Chromatographic peaks were integrated using Class LC10 work station (Shimadzu).

2.3. Stock and standard solution preparation

Individual stock solutions containing 200 µg/ml of I, III, IV and V and 400 µg/ml of II were prepared in DMSO. Mixed standard solutions of I–V were prepared in DMSO between 100 ng/ml and 10 µg/ml by adding specific volumes of stock solutions to individual volumetric flasks. Working stock solution containing I, III–V (40 µg/ml) and II (80 µg/ml) in DMSO was prepared by mixing 1 ml each of individual stock solutions in a 5 ml volumetric flask. Calibration standards of I–V were prepared in human plasma by spiking with a fixed volume of working stock solution and then serially diluting to get 10–500 ng/ml concentrations for I, III–V and 20–1000 ng/ml for II. All spiked plasma solutions were stored at −30°C until analyzed by HPLC.

2.4. Sample preparation

Blank or spiked plasma (1 ml) was basified with 0.2 ml of 1 M borate buffer (pH 10.5). After adding 10 µl of mebendazole as I.S. (10 µg/ml), it was vortex mixed with 4 ml diethyl ether for 1 min. The sample was centrifuged at 1000 g for 10 min at 4°C and the supernatant was separated after snap freezing the aqueous layer in liquid nitrogen. Extraction was repeated with another 4-ml aliquot of ether. Combined ether extract was evaporated under reduced pressure in Speed Vac concentrator (Savant Instrument, Farmingdale, NY, USA) below 40°C and the residue was reconstituted in 100 µl of DMSO for injection onto the HPLC system.

2.5. Validation

2.5.1. Calibration curve
Standard curves drawn from seven different batches were pooled and both pooled and individual data sets were used to select the calibration model. Microsoft Excel software [2] was used to plot the calibration curve by linear regression with different weights ($1/x$, $1/x^2$ and $1/\sqrt{x}$).

2.5.2. Recovery
Recoveries of I–V from spiked plasma samples were calculated by comparing the peak heights at all concentration levels with the standard curve obtained by analyzing the corresponding standard dilutions in DMSO, injected directly.

2.5.3. Accuracy and precision
Accuracy and precision of the assay method were studied at low (lowest limit of quantitation, LLOQ): 10 ng/ml for I, III–V and 20 ng/ml for II; medium: 100 ng/ml for I, III–V and 200 ng/ml for II and high: 500 ng/ml for I, III–V and 1000 ng/ml for II concentration levels. Triplicate plasma samples at each of the three concentration levels were processed and analysed in each run and four such batches were assessed. Variations in accuracy and precision were expressed as % bias and relative standard deviation (R.S.D.), respectively [3].

Acceptance limits of ±20% at the lowest limit of quantitation (LLOQ) and ±15% at other concentrations in the calibration range were used for the validation [4].

2.5.4. Effect of freeze–thaw (f–t) cycles on the stability of UMF-078 and its metabolites in plasma
Low (LLOQ), medium and high concentration samples (1 ml) were stored in glass tubes at −30°C. One set of three concentrations in triplicate was analysed on day 1 (no f–t cycle) and other similar sets were analysed after one, two and three f–t cycles. Thawing was achieved by keeping the tubes at room temperature for 30 min. Concentrations obtained from the spiked plasma samples not subjected to f–t cycles were considered 100% and those calculated on analysis after subsequent f–t cycle were compared with the initial concentrations.

2.5.5. In-process stability of UMF-078 and its metabolites in the dry plasma extract at −30°C
The effect of storing the dry residue after extraction at −30°C on the stability of I–V was studied at low, medium and high concentration levels as mentioned above. Replicate samples of plasma were extracted and one set (comprising triplicates of each concentration level) was reconstituted with DMSO and analyzed on the same day (day 1). The other tubes containing dry residue were sealed and stored

at −30°C. These samples were reconstituted and analyzed on days 3 and 4 after extraction. Data analysis was carried out as described for f–t cycles.

3. Results and discussion

3.1. Optimization of sample clean-up and chromatographic conditions

With the chromatographic conditions reported by Navratnam et al. [1] compound I and its metabolites were not sufficiently resolved for quantitation. Therefore, the pH and molarity of the buffer, the ratios of the organic modifiers and the gradient conditions were optimised. Optimization of sample clean-up technique was also done to get rid of the interfering endogenous substances without sacrificing the recoveries of the analytes. Extraction of the blank/spiked plasma with diethyl ether containing 5% isopropyl alcohol (IPA) improved the recoveries of I and II by 11 and 16%, respectively, but extracted many polar interfering substances from matrix. To remove these polar substances, back-extraction with various organic solvents like *n*-hexane, ethyl acetate (EA) and their admixtures were tried after acidification of the plasma extract. *n*-Hexane was unable to remove most of these polar substances extracted by IPA, whereas on back-extraction with EA more than 90% of the drug was lost along with the metabolites [5]. The molarity and the type of basifying agent added to matrix also affected the recovery of the analytes [1]. With 0.2 ml of 1 *M* borate buffer (pH 10.5) instead of KOH there was a 30 and 40–45% increase in the recoveries of the parent drug and metabolites, respectively. Addition of 2 ml of 0.1 *M* borate buffer increased the enrichment of endogenous interfering substances with an increasing tendency of emulsification on vortex mixing. Emulsification of the two phases was a serious problem encountered when 2 ml of 0.05 *M* KOH was used [5]. When 100 µl of 1 *M* KOH was used there was a 3–5% decrease in the recovery of all the analytes but it then minimised the emulsification.

The effects of pH and molarity of the buffer in the mobile phase on the peak heights of the analytes were also studied [5]. There was a negative drift in the baseline and the peaks of II and III merged with I

and IV at pH 3.0, respectively . At higher pH (5 and 6) peaks of II and III split with a 10–35% decrease in the peak heights of I, II and III. This phenomenon could be attributed to the decrease in solubility of the analytes in the mobile phase at higher pH (the analytes being weakly basic in nature). The retention times of I and II shifted from 7.6 and 8.6 min, respectively to 6.8 and 7.9 min, respectively when molarity of the buffer was decreased from 50 to 25 m*M*. The retention times of III, IV and V were unaffected by the molarity of the buffer. However, there was an 8–10% decrease in the peak heights of all analytes. An increase in molarity from 50 to 75 m*M* resulted in the precipitation of the buffer salts in the mobile phase at 55% concentration of the organic modifier.

The linear gradient elution (ranging from 30–55%) was employed to accommodate resolution of all the analytes compared with the step gradient elution employed earlier [1]. The effect of organic modifiers like *N,N*-dimethyloctylamine (DMOA) and tetrahydrofuran (THF) on the resolution of analytes was also studied. Addition of 0.1% (v/v) DMOA to the mobile phase decreased the retention times of I and II by 18.8 and 17%, respectively while increasing that of III by 8%. Whereas, the peak heights increased by 6.8% (I), 17% (II) and decreased by 15% for III. Incorporation of THF (1–2%, v/v) to the mobile phase resulted in poor resolution of the analytes.

During optimization of mobile phase compositions, the best results in terms of resolution, sensitivity and run time were obtained using potassium dihydrogenorthophosphate (pH 4, 50 m*M*) in pump A and 25% (v/v) acetonitrile in methanol in pump B. The extraction procedure and the chromatographic conditions were suitable for the quantitative analysis of the analytes I–V and the I.S. The plasma endogenous substances did not interfere with the elution of any of the analytes (Fig. 2A). The retention times of analytes I–V and the I.S. were 8.6, 7.5, 10.7, 12.2, 15.7 and 14.5 min, respectively. Fig. 2 illustrates the chromatograms of drug free plasma, analytical standard containing I–V and plasma spiked with I, its four metabolites and the I.S.

The calibration model was selected based on the analysis of both individual and pooled calibration data by linear regression with and without intercepts
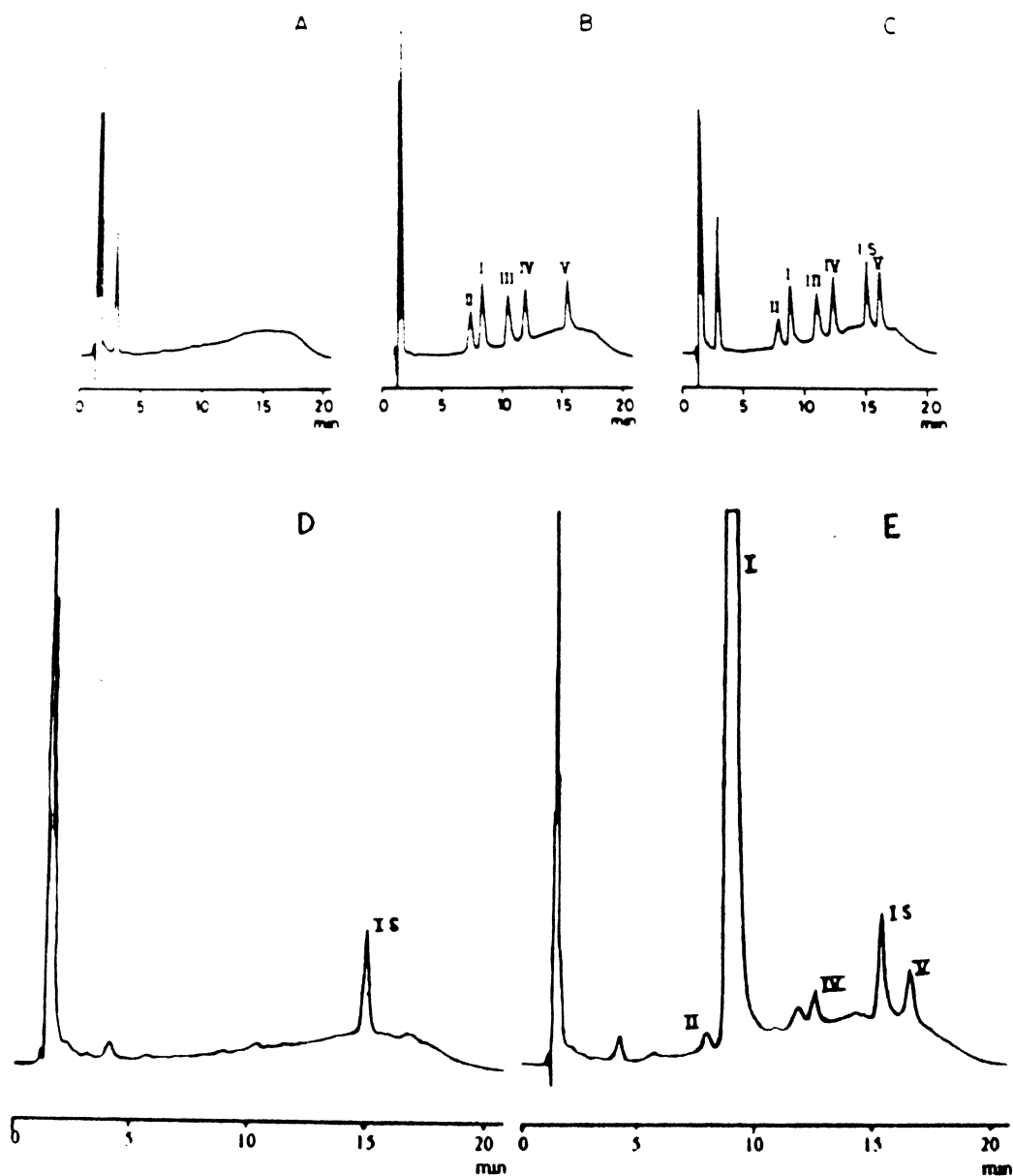
Fig. 2. Representative chromatograms of (A) blank plasma, (B) analytical standard containing 1000 ng/ml of I, III–V and 2000 ng/ml of II, (C) spiked plasma containing 100 ng/ml of I, III–V and 200 ng/ml of II, (D) drug free rat serum and (E) rat serum after an oral dose of hydrochloride salt (UMF-289) equivalent to 100 mg/kg of I.

and weighting factors ($1/x$, $1/x^2$ and $1/\sqrt{x}$). The equation passing through origin ($y=mx$, where y is peak height, x is concentration, and m is slope) without any weighting factor was found to be the best fit for the calibration data of each of the analytes. The analysis of validation samples in four batches also confirmed the suitability of the model and hence this equation was used to calculate

Table 1
Mean recoveries of I and its metabolites from spiked human plasma

Concentration (ng/ml)	Absolute recovery (mean±SD, n=7) (%)				
	I	II	III	IV	V
10	75±4.7	61±6.3	78±6.7	96±0.3	115±7.1
20	76±3.0	60±3.3	79±3.5	100±8.2	116±2.6
50	77±3.2	60±2.7	82±2.6	99±5.0	118±10.3
100	80±3.1	60±3.4	85±4.0	102±5.6	118±7.4
200	83±3.3	60±3.3	86±3.5	101±5.3	120±11.5
500	84±4.8	60±3.2	88± 3.2	104±4.0	124±9.0

recovery, accuracy, precision and stability of each of the analytes.

3.2. Recovery

Mean recoveries from plasma at all concentration levels are summarized in Table 1. The intra-batch variations in recoveries for I–V were found to be as 5.49, 4.66, 6.31, 4.28 and 5.66%, respectively. The mean recoveries of I (79.1±4.8%), IV (100.48±5.5%) and V (118.5±8.4%) were found to be slightly higher compared to 69.9±4.7%,

85.6±4.4% and 85.1±6.0%, respectively reported earlier [1].

3.3. Accuracy and precision

Inter- and intra-batch precision and accuracy were estimated in spiked plasma samples analysed in replicates on three days. The overall % bias and precision at the three concentrations are presented in Tables 2 and 3. The results show that the method is accurate and the bias is within the acceptance limits

Table 2
Intra-batch accuracy and precision of I and its metabolites in spiked human plasma

Analytes	Low (10 ng/ml)		Medium (100 ng/ml)		High (500 ng/ml)	
	% Bias	RSD (%)	% Bias	RSD (%)	% Bias	RSD (%)
I	−11.6	3.2	−5.1	3.8	−0.1	2.9
II[a]	−4.14	7.0	−0.1	3.8	−1.0	3.0
III	−11.7	3.8	−3.1	3.8	0.7	2.5
IV	−11.9	4.8	−4.4	4.7	−5.0	3.7
V	−5.13	3.5	−2.3	3.6	0.7	3.5

[a] Indicates low (20 ng/ml), medium (200 ng/ml) and high (1000 ng/ml); (n=3).

Table 3
Inter-batch accuracy and precision of I and its metabolites in plasma

Analytes	Low (10 ng/ml)		Medium (100 ng/ml)		High (500 ng/ml)	
	% Bias	RSD (%)	% Bias	RSD (%)	% Bias	RSD (%)
I	−11.1	5.0	−5.1	10.7	2.1	5.8
II[a]	−5.0	11.6	−7.2	15.8	−2.1	7.8
III	−11.5	2.4	−3.6	10.3	0.6	9.6
IV	−14.3	11.0	−5.2	5.2	−4.9	3.1
V	−5.1	3.2	−3.1	5.8	0.1	13.4

[a] Indicates low (20 ng/ml), medium (200 ng/ml) and high (1000 ng/ml); (n=3).

of ±20% at LLOQ and ±15% at all other concentrations. Similarly the RSD for various analytes was within ±15% at all the concentration levels studied except that the RSD for II at medium level was 15.8%.

3.4. Effect of f–t cycles on the stability of UMF-078 and its metabolites

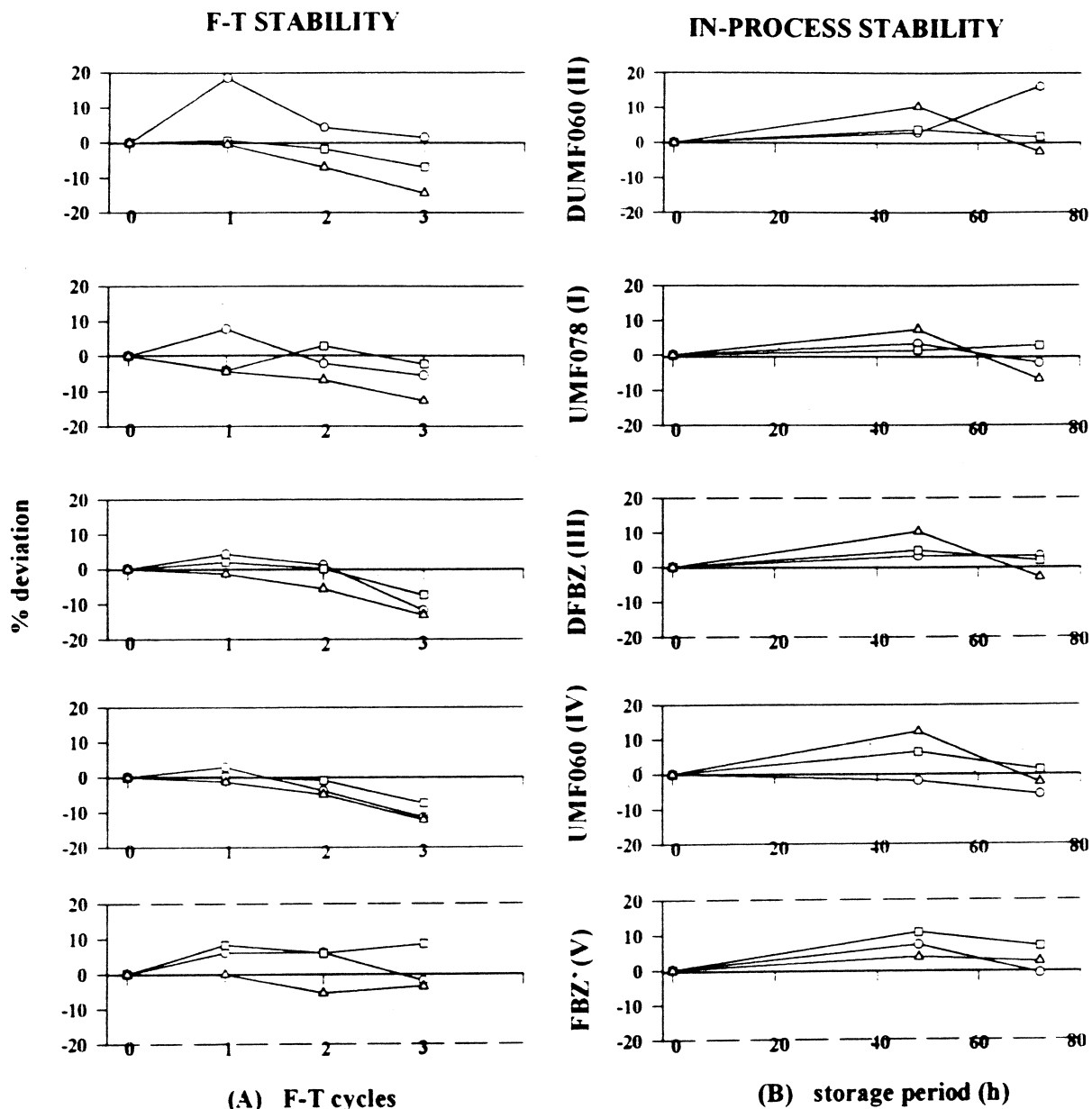The deviations observed after one, two and three f–t cycles were within 20% at LLOQ and 15% at all

Fig. 3. Effect of freeze–thaw cycles (A) and storage of the residue at −30°C (B) on the stability of the analytes (○ – low, 10 ng/ml for I, III–V and 20 ng/ml for II; □ – medium, 100 ng/ml for I, III–V and 200 ng/ml for II; and △ – high, 500 ng/ml for I, III–V and 1000 ng/ml for II).

other concentration levels. These deviations represent effect of f–t cycles on stability and also the inherent inter-/intra-batch variations. A trend demonstrating loss of I–IV after two f–t cycles was observed (Fig. 3A), indicating that the repeated freeze–thaw cycles should be avoided.

3.5. In process stability of UMF-078 and its metabolites in the dry extract at −30°C

It was found that I and its metabolites were stable in the dry extract stored at −30°C up to 72 h after sample preparation at all the three concentrations (Fig. 3B). The deviations were within 17% for LLOQ and less than 13% at medium and high concentration levels for all the compounds (highest variations 12.3% at high level for IV, over a period of 72 h). These observed variations were comparable to intra-/intra-batch precision. No trend was observed in the read concentrations after 48 h and 72 h of storage.

4. Application

A hydrochloride salt of UMF-078 was prepared to enhance its aqueous solubility and a solution formulation for oral administration to rats was prepared by dissolving the salt (equivalent to 25 mg/ml of free base) in water. The assay method described herein was applied to determine the concentration–time profile of I, II, IV and V in young healthy male Sprague–Dawley rats after administering a single oral dose of UMF-289 (hydrochloride salt of I) equivalent to 100 mg/kg of I. Serial blood samples were collected post oral dose and serum was harvested and stored at −30°C until analysis. The method was sensitive enough to follow I, II, IV and V up to 168 h post-dose. Typical representative chromatograms of blank and treated rat serum are given in Fig. 2. The chromatograms did not exhibit any interferences from endogenous components of rat serum at regions of elution of any of the analytes. However an unknown peak appeared between I and IV in the treated serum sample which could be an additional metabolite of I. The concentration–time data of I, II, IV and V after an oral dose equivalent to

Table 4
Serum concentration–time profile of I and its metabolites on oral administration of hydrochloride salt (UMF-289) equivalent to 100mg/kg of I in rat

Time (h)	Concentration (ng/ml)			
	I	II	IV	V
0.5	3400	NQ[a]	19	37
3.0	5790	90	51	86
8.0	6004	103	87	112
24	8956	65	134	138
72	2918	52	46	88
96	883	–	38	71
168	211	46	37	20

[a] NQ: Not quantitated (below LLOQ), $n=4–5$.

100 mg/kg of I are given in Table 4. The HPLC method was developed and validated in pooled human plasma from a local blood bank because it was felt unnecessary and unethical to sacrifice about 50 rats to collect blank plasma/serum pool for development and validation of this assay method.

However, since the method was applied to rat serum matrix, the matrix interference, recoveries of analytes and variations in accuracy and precision were assessed using triplicate of blank and spiked rat serum samples prior to analysis of unknowns. The recoveries of I and its metabolites at low, medium and high concentrations in rat serum were slightly (10%) less than human serum. Hence the calibration standards were plotted in rat serum for analysis of rat samples in order to take care of the differences in recoveries of the analytes between human plasma and rat serum. The serum samples were analyzed within one week of collection and they were not subjected to more than two f–t cycles. Spiked QC samples in rat and human serum did not show any loss of analytes during a six-month study period at −30°C.

5. Conclusions

Optimum separation of all analytes was achieved by gradient elution. Variations in recoveries of I and its metabolites were within the acceptance limits of ±20% at LLOQ and ±15% at all other concentrations. The method is accurate and the bias and

RSD were within the acceptance limits of ±20% at LLOQ and ±15% at higher concentrations. Storage of I and its four metabolites in serum up to six months or in dry plasma extract up to 72 h at −30°C showed no indications of degradation. There was no change in the read concentrations of any of the analytes up to one freeze–thaw cycle, but a trend of decreasing concentrations for I–IV was observed subsequent to two and three f–t cycles, indicating that the analysis of the frozen plasma samples after two f–t cycles should be avoided.

Acknowledgements

References

[1] S. Ramnathan, N.K. Nair, S.M. Mansor, V. Navratnam, J. Chromatogr. B 655 (1994) 269.

[2] Microsoft Excel, Version 5.0, Microsoft Inc., USA

[3] C. Hartmann, W. Penninckx, Y.V. Heyden, P. Vankeerberghen, D.L. Massart, R.D. McDowell, in: H.H. Blume, K.K. Midha (Eds.), Experience with Chromatographic Methods–Europe, in Bio'94, Bio-International 2, Bioavailability, Bioequivalence and Pharmacokinetic Studies, Medpharm Scientific Publishers, Stuttgart, Germany, 1995, pp. 331–346.

[4] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, J. Pharm. Sci. 81 (1992) 309.

[5] B.V. Atul, J.K. Paliwal, R.C. Gupta, J. Pharm. Biomed. Anal. (1998) in press.