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Determination of a potential antiasthmatic compound, tibenelast, and its application to phase I clinical trials

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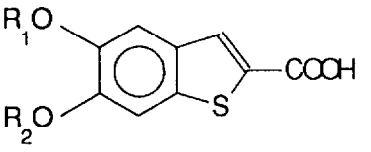
ABSTRACT

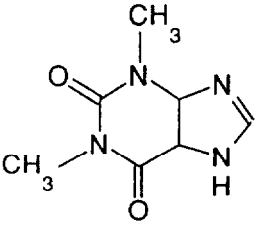
A sensitive and selective high-performance liquid chromatographic (HPLC) assay was developed for the determination of tibenelast, 5,6-diethoxybenzo[*b*]thiophene-2-carboxylic acid, in plasma and urine. The plasma assay involves protein precipitation with 4% trichloroacetic acid, while the urine assay is an automated solid-phase extraction procedure that utilizes the Waters Millilab™ workstation. The analysis was achieved by reversed-phase HPLC with ultraviolet detection at 313 nm. The quantitation limit of the assay was 50 ng/ml in plasma and 100 ng/ml in urine. The intra-day coefficient of variation for the plasma analysis was between 2.2 and 8.4%, while the overall inter-day coefficient of variation was 5.5 and 6.0% for the high and low calibration curves, respectively. The intra-day coefficient of variation for the urine analysis was between 0.3 and 3.0%, while the inter-day coefficient of variation was 2.1% for both the low and high validation samples. The assay methodology has been used in the evaluation of samples from pharmacokinetic and clinical safety studies.

INTRODUCTION

The sodium salt of 5,6-diethoxybenzo[*b*]thiophene-2-carboxylic acid (tibenelast sodium) has been identified as a potential anti-asthma compound in rat, guinea pig, dog and sheep. The preclinical pharmacology has shown tibenelast to be an orally active bronchodilator [1,2]. Tibenelast was 30 times more potent than aminophylline in neutrophils for the inhibition of superoxide anion release [1]. It appears to inhibit phosphodiesterase activity in bronchial tissue of anesthetized guinea pig without altering heart rate, blood pressure



COMPOUND	Number	R_1	R_2
Tibenelast	I	CH_3CH_2	CH_3CH_2
Metabolite 1	II	H	CH_3CH_2
Metabolite 2	III	$HOCH_2CH_2$	CH_3CH_2
Metabolite 3	IV	H	H
Urine internal standard	V	$CH_3CH_2CH_2$	CH_3
Plasma internal standard	VI	CH_3CH_2	$CH_3CH_2CH_2$



Theophylline

Fig. 1. Structures of tibenelast (5,6-diethoxybenzo[*b*]thiophene-2-carboxylic acid), metabolite 1 (5-hydroxy-6-ethoxybenzo[*b*]thiophene-2-carboxylic acid), metabolite 2 [5-(2-hydroxyethoxy)-6-ethoxybenzo[*b*]thiophene-2-carboxylic acid], metabolite 3 (5,6-dihydroxybenzo[*b*]thiophene-2-carboxylic acid), the internal standard for the urine assay (5-propoxy-6-methoxybenzo[*b*]thiophene-2-carboxylic acid), the internal standard for the plasma assay (5-ethoxy-6-propoxybenzo[*b*]thiophene-2-carboxylic acid) and theophylline.

and myocardiac contractility at 1.0 mg/kg when compared to aminophylline [3]. Tibenelast is a thiophene derivative in contrast to aminophylline which is a xanthine derivative (Fig. 1). The clinical development of tibenelast required an analytical procedure for the determination of tibenelast in plasma and urine.

In anticipation of clinical trials, a high-performance liquid chromatographic (HPLC) method has been developed and validated for the determination of tibenelast in plasma and urine. Sample preparation was developed to be rapid and reproducible. The plasma assay involves the precipitation of plasma proteins with 4% (w/v) trichloroacetic acid (TCA), centrifugation and injection into the HPLC system. The urine analysis utilizes a Waters Millilab™ workstation which performs an automated solid-phase extraction procedure. The assay procedures were used to determine plasma and urine concentrations of tibenelast in samples from phase I clinical trials.

EXPERIMENTAL

Reagents

Acetonitrile and methanol, whether used for sample preparation or mobile phase, were obtained from Burdick and Jackson (Muskegon, MI, U.S.A.). The 1 *M* acetic acid was prepared by diluting 57.4 ml of glacial acetic acid (Mallinckrodt Chemical Works, Paris, KY, U.S.A.) to 1 l with deionized water. The 4% (w/v) TCA was prepared by adding 4 g of TCA (Sigma, St. Louis, MO,

U.S.A.) to a 100-ml volumetric flask and filling to the mark with deionized water. Human plasma and urine were obtained from either volunteers at the Lilly Laboratory for Clinical Research or from Biological Specialities Corporation (Lansdale, PA, U.S.A.).

Tibenelast, radiolabeled tibenelast, the plasma and urine internal standards and authentic standards of known animal metabolites were synthesized at Eli Lilly and Company (Indianapolis, IN, U.S.A.) (Fig. 1). These compounds were identified by NMR, mass spectrometry, UV detection and elemental analysis and shown to be pure by chromatographic methods.

Apparatus

The HPLC system for the plasma analysis contained a Waters M45 pump (Milford, MA, U.S.A.), a Waters WISP 712 autoinjector, a Waters data module and a Waters system controller. Detection was achieved with a Kratos Spectraflow 757 UV detector (Foster City, CA, U.S.A.) set at 313 nm with a 0.01 absorbance range. The HPLC was equipped with an Ultrasphere ODS (15 cm×4.6 mm I.D., 5-μm packing material) column. The mobile phase (1 *M* acetic acid–acetonitrile, 55:45, v/v) was delivered at a flow-rate of 1.4 ml/min. The data acquisition was sent to a local integrator and to a Hewlett-Packard (Avondale, PA, U.S.A.) HP1000 data system.

The HPLC system for the urine analysis contained a Beckman 126 programmable solvent module (San Ramon, CA, U.S.A.), a Waters WISP 712 autoinjector and a Shimadzu Altex Model C-R1A integrator (Kyoto, Japan). Detection was achieved with a Kratos Spectraflow 783 UV detector set at 313 nm with a 0.01 absorbance range. The HPLC was equipped with an Ultrasphere ODS (15 cm×4.6 mm I.D., 5-μm packing material) column. The mobile phase (1 *M* acetic acid–acetonitrile, 70:30, v/v) was delivered at a flow-rate of 1.5 ml/min. The data acquisition was sent to a local integrator and to a Hewlett-Packard HP1000 data system.

The solid-phase extraction of tibenelast from urine was performed on a Waters Millilab workstation equipped with C_{18} solid-phase extraction columns and the following solvents: methanol, deionized water, methanol–water (50:50, v/v) and acetonitrile.

Standard solutions

A stock solution of tibenelast sodium (Lot No. L04DQ13) was prepared by dissolving 5.41 mg into a 5-ml volumetric flask and filling to the mark with a methanol–water (20:80, v/v) solution. This produced a solution equivalent to 1.0 mg/ml free acid.

Internal standard (I.S.) solutions (10 and 2 μg/ml) for the plasma assay (5-ethoxy-6-propoxybenzo[*b*]thiophene-2-carboxylic acid; Lot No. M82-CC5-126) were prepared in acetonitrile.

A 2 μg/ml I.S. solution for the urine assay (5-propoxy-6-methoxy-

benzo[*b*]thiophene-2-carboxylic acid; Lot No. M82-T28-237) was prepared in water. Plasma standards were prepared at concentrations of 50–1000 ng/ml and 0.5–10 μg/ml. Samples used for the validation of the assay were prepared at concentrations of 0.075, 0.75 and 7.5 μg/ml.

The urine standards were prepared at concentrations between 100 and 1000 ng/ml. Samples used for the validation of the assay were prepared at concentrations of 150 and 750 ng/ml. All samples were frozen at −20°C until assayed.

Extraction procedures

A 0.2-ml volume of plasma was transferred into a 15-ml siliconized glass disposable test tube [4]. A 0.1-ml aliquot of the 10 μg/ml I.S. solution was added to the test tube if the sample was in the concentration range 0.5–10 μg/ml. A 0.05-ml aliquot of the 2 μg/ml I.S. solution was added to the test tube if the sample was in the concentration range 50–1000 ng/ml. A 0.2-ml volume of the 4% (w/v) TCA solution was then added to ensure precipitation of the plasma proteins. The samples were mixed for 15 s, left for 30 min and centrifuged at 1000 *g* for 10 min. A 100-μl aliquot (for samples in the concentration range 0.5–10 μg/ml) of the supernatant was transferred to a new siliconized glass disposable test tube, and 500 μl of mobile phase (1.0 *M* acetic acid–acetonitrile, 55:45, v/v) were added to the sample. The sample was mixed, and 100–150 μl were injected into the HPLC system. All of the supernatant was transferred to a new siliconized glass disposable test tube if the sample was in the concentration range 50–1000 ng/ml. A 150-μl volume of mobile phase (1.0 *M* acetic acid–acetonitrile, 55:45, v/v) was added to the sample. After mixing, 200 μl were injected into the HPLC system.

A 1.0-ml volume of urine was transferred into a 15-ml siliconized glass disposable test tube. A 0.25-ml volume of the 2 μg/ml I.S. solution was added to the test tube. The sample was mixed for 15 s and placed on the Waters Millilab workstation for analysis. The Millilab workstation performed an automated solid-phase extraction which involved the following procedure.

Column conditioning. The column was washed with two 2.0-ml aliquots of methanol, followed by one 2.0-ml aliquot of water.

Sample application. A 1.0-ml sample was aliquoted from the sample tube onto the C_{18} solid-phase extraction column.

Sample clean-up. The solid-phase extraction column was washed twice with 2.0 ml of deionized water, followed by 4.0 ml of a methanol–water (50:50, v/v) solution.

Sample elution. The components were eluted with 1.0 ml of acetonitrile. The eluent was dried with nitrogen and heat. The sample was reconstituted in 0.25 ml of mobile phase (1 *M* acetic acid–acetonitrile, 70:30, v/v), mixed and injected into the HPLC system for analysis.

Precision, accuracy, linearity, reproducibility and recovery

Chromatographic data acquisitions and calculations were performed by the Hewlett-Packard laboratory automation system. A linear least-squares regression analysis of the peak-height ratio of tibenelast to the appropriate I.S. versus added concentration to drug-free human plasma or urine served as daily instrument standardization. The slope, intercept, correlation coefficient and the relative standard deviation (R.S.D., %) of the calibrators from the linear regression line were calculated.

To test for the precision, accuracy, linearity and reproducibility of the method, a standard curve and five validation samples at two different concentrations were run on three consecutive days in plasma and urine. The ranges of the plasma curves were 50.0–1000.0 ng/ml and 0.5–10 μg/ml. The range of the urine curve was 100–1000 ng/ml.

The validation statistics were calculated using the Symphony program from Lotus Development (Cambridge, MA, U.S.A.). The evaluation of precision, accuracy and reproducibility was based on the calculation of relative error (R.E., %) and standard deviation [R.S.D. (%) or coefficient of variation (C.V., %)], respectively. Linearity was judged by visual inspection, the correlation coefficient (r) and the R.S.D. (%) of the data from the linear regression line. Recovery of tibenelast was determined by the use of radioactivity.

Biomedical application

A phase 1 clinical trial was designed to investigate the pharmacokinetics of tibenelast when given as a single dose and during multiple dosing to seven healthy male volunteers. Each volunteer was dosed with 100 mg of tibenelast, and blood samples (7 ml) were drawn into heparinized vacutainer tubes at 0, 0.25, 0.5, 1, 2, 4, 8, 12, 24, 36, 48 and 72 h after drug administration. Five days following the single dose, each volunteer was dosed with 50 mg every 12 h (q12h) for five days. Blood sampling (7 ml) was carried out after the last dosing interval at 0, 0.25, 0.5, 1, 2, 4, 8 and 12 h. Following the 50-mg q12h regimen, each volunteer received 100 mg q12h for five days and then 150 mg q12h for five days. Blood sampling was carried out as in the 50-mg q12h dosing regimen. The resulting plasma samples were stored frozen at −20°C until assayed. Daily urine samples were collected four days preceding the 100-mg single dose and throughout the study. The volumes of the samples were measured, and 250-ml aliquots of the samples were frozen and kept at −20°C until assayed.

RESULTS AND DISCUSSION

Typical chromatograms for the analysis of tibenelast in human plasma and urine can be seen in Figs. 2 and 3. The drug-free samples are without interferences at the retention times of tibenelast and the internal standards. The retention times of tibenelast and the internal standard are 4.8 and 8.5 min, re-

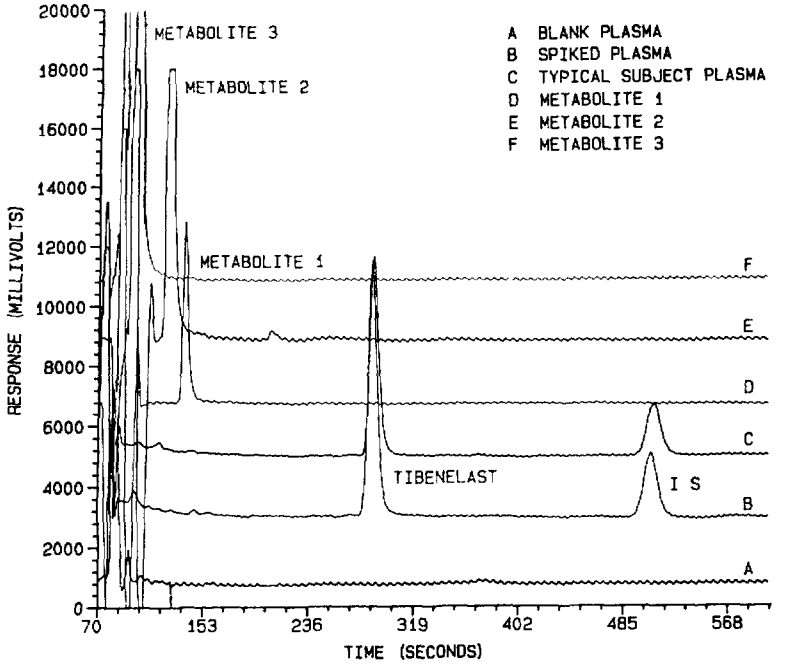


Fig. 2. Chromatograms from the plasma analysis of tibenelast. Extracts of (A) a blank (drug-free) human plasma sample, (B) a drug-free plasma sample spiked with 7.5 μg/ml tibenelast and 2.1 μg of the internal standard, (C) a plasma sample from a volunteer after receiving a 100-mg oral dose of tibenelast, (D) a reference standard of metabolite 1, (E) a reference standard of metabolite 2 and (F) a reference standard of metabolite 3.

spectively, in the plasma analysis and 14.3 and 16.8 min, respectively, in the urine analysis. The internal standards were selected because of their structural similarity to tibenelast.

The high plasma concentration range was linear between 0.5 and 10 μg/ml (Table I). The mean equation that described the line was $y = 0.183(\pm 0.002)x - 0.007(\pm 0.0003)$ with a correlation coefficient of 0.9997 ($y = mx + b$) and an R.S.D. from the line of 2.6% (± 1.4). The low plasma concentration range was linear between 50 and 1000 ng/ml (Table II). The mean ($n = 6$) equation that described the line was $y = 0.0017(\pm 0.0001)x + 0.0018(\pm 0.0067)$ with a correlation coefficient of 0.9998 and an R.S.D. of 2.2% (± 0.07). The intra-day C.V. for the low concentration range was between 4.3 and 8.36%. The overall inter-day C.V. for the high and low concentration ranges was 5.5 and 6.0%, respectively. The recovery for the high plasma concentration range was between 38 and 56% and for the low plasma concentration range between 27 and 36%. The assay characterization demonstrated that these recoveries do not inhibit the determination

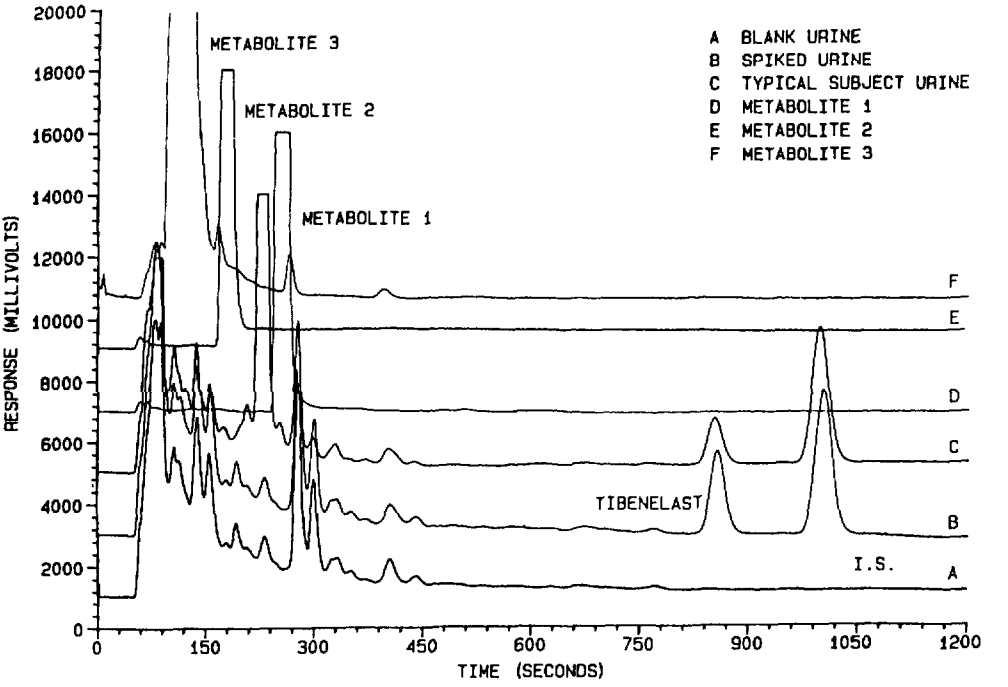


Fig. 3. Chromatograms from the urine analysis of tibenelast. Extracts of (A) a blank (drug-free) human urine sample, (B) a drug-free urine sample spiked with 750 ng/ml tibenelast and 1100 ng of the internal standard, (C) a urine sample from a volunteer after receiving a 100-mg oral dose of tibenelast, (D) a reference standard of metabolite 1, (E) a reference standard of metabolite 2 and (F) a reference standard of metabolite 3.

of tibenelast. This is probably due to the selection of a structural analogue as the internal standard.

The urine concentration range was linear between 100 and 1000 ng/ml (Table III). The mean ($n=3$) equation that described the line was $y=0.0019(\pm 0.0001)x-0.0016(\pm 0.0014)$ with a correlation coefficient of 0.9999 and an R.S.D. of 1.3% (± 0.7). The intra-day C.V. for the 750 ng/ml sample was between 1.2 and 3.0%. The intra-day C.V. for the low concentration control was between 0.3 and 1.2%. The overall inter-day C.V. for both the high and low controls was 2.1%. The pH of the urine must be below 6.7 for the solid-phase extraction to work. The pH may be lowered by the addition of 10 μl of glacial acetic acid to the urine sample. The recovery of tibenelast from urine was between 75 and 77%. The application of the solid-phase extraction method, applied to plasma samples, may increase the recovery of tibenelast and thereby increase the sensitivity of the method. This approach has not been evaluated because increased sensitivity was not necessary for the phase I clinical trials.

The analysis of tibenelast in plasma and urine was shown to be selective for tibenelast. Three isolated animal metabolites have been identified and synthe-

TABLE I

ASSAY VALIDATION STATISTICS OF THE HIGH PLASMA CONCENTRATION RANGE

R.S.D. (%) = (S.D./mean) × 100. R.E. (%) = [(mean/truth) × 100] − 100.

Run	Concentration found (μg/ml)					Mean ± S.D. (μg/ml)	Imprecision (R.S.D., %)	Inaccuracy (R.E., %)
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5			
Assay validation sample of 0 75 μg/ml								
Intra-day								
170	0.76	0.74	0 76	0.80	0.81	0.77 ± 0.03	3.47	3.17
176	0.75	0.76	0.72	0.76	0.73	0.74 ± 0.02	2.31	−1.04
179	0.69	0.69	0.71	0.70	0.74	0.71 ± 0.02	2.52	−5.69
Inter-day						0.74 ± 0.03	4.62	−1.19
Assay validation sample of 7.50 μg/ml								
Intra-day								
170	6.96	6.52	7.71	7.08	7.18	7.09 ± 0.43	6.05	−5.48
176	6.72	6.48	6.72	7.71	6.58	6.84 ± 0.50	7 26	−8.78
179	7.07	6.93	7.33	7.23	7.11	7.13 ± 0.15	2.16	−4.87
Inter-day						7.02 ± 0.38	5.47	−6.38

TABLE II

ASSAY VALIDATION STATISTICS OF THE LOW PLASMA CONCENTRATION RANGE

R.S.D. (%) = (S.D./mean) × 100. R.E. (%) = [(mean/truth) × 100] − 100.

Run	Concentration found (ng/ml)					Mean ± S.D. (ng/ml)	Imprecision (R.S.D., %)	Inaccuracy (R.E., %)
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5			
Assay validation sample of 750 ng/ml								
Intra-day								
194	739.1	651.1	665.4	658.4	782.7	699.34 ± 58.49	8.36	−6.75
196	672.7	697.1	756.7	688.6	698.8	702.78 ± 31.86	4.53	−6.30
197	732.9	698.3	690.7	677.1	651.2	690.94 ± 29.92	4.34	−7.99
Inter-day						697.39 ± 39.43	5.65	−7.02
Assay validation sample of 75 ng/ml								
Intra-day								
198	72.6	75.4	63.8	68.7	71.7	70.44 ± 4.40	6.24	−6.08
199	69.5	70.6	78.0	65.8	70.5	70.88 ± 4.44	6.27	−5.49
200	73.0	78.8	65.4	70.1	72.3	71.92 ± 4.85	6.75	−4.11
Inter-day						71.08 ± 4.28	6.02	−5.23

sized. When analyzed by these chromatographic systems they were shown not to interfere with the determination of tibenelast. The metabolites are 5-hydroxy-6-ethoxybenzo[*b*]thiophene-2-carboxylic acid (metabolite 1), 5-(2-hydroxyethoxy)-6-ethoxybenzo[*b*]thiophene-2-carboxylic acid (metabolite 2)

TABLE III

ASSAY VALIDATION STATISTICS OF THE URINE CONCENTRATION RANGE

R.S.D. (%) = (S.D./mean) × 100. R.E. (%) = [(mean/truth) × 100] − 100.

Run	Concentration found (ng/ml)					Mean ± S.D. (ng/ml)	Imprecision (R.S.D., %)	Inaccuracy (R.E., %)
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5			
Assay validation sample of 150 ng/ml								
Intra-day								
20	158.1	157.0	157.6	154.5	153.9	156.2 ± 1.9	1.2	4.1
21	149.8	147.1	149.7	148.0	151.4	148.7 ± 1.3	0.9	−9.9
22	152.1	152.7	152.6	152.3	151.7	152.3 ± 0.4	0.3	1.5
Inter-day						152.6 ± 3.3	2.1	1.7
Assay validation sample of 750 ng/ml								
Intra-day								
20	742.6	726.2	770.8	770.0	727.6	751.4 ± 22.6	3.0	0.2
21	746.7	752.0	741.1	731.7	N.S.[a]	742.9 ± 8.7	1.2	−1.0
22	741.7	737.9	474.3	739.8	720.6	737.5 ± 10.1	1.4	−1.7
Inter-day						744.0 ± 15.6	2.1	−0.8

[a]Sample lost in processing.

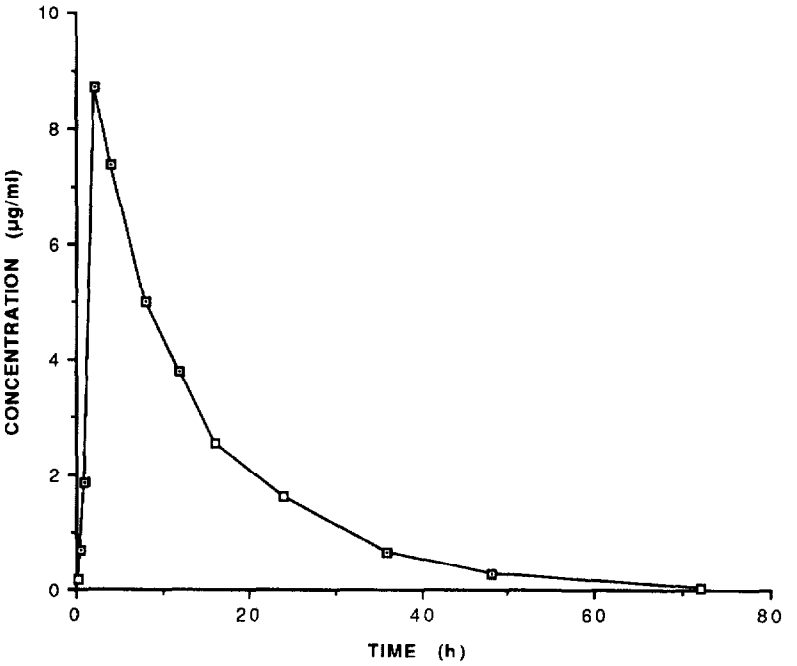


Fig. 4. Tibenelast plasma concentration versus time profile after a single oral dose of 100 mg tibenelast in man.

and 5,6-dihydroxybenzo[*b*]thiophene-2-carboxylic acid (metabolite 3) (Fig. 1). Tibenelast has been determined in the presence of theophylline, albuterol and isoproterenol. These drugs do not interfere with the determination of tibenelast in plasma.

The stability of tibenelast was examined under conditions that may be experienced during the course of a clinical study. These conditions include three-month storage of samples at −20°C, benchtop stability (sample drawing and handling procedures) and assay stability (repetitive freeze–thaw cycles and the stability in a WISP vial in case of instrument failure). The stability of a sample stored at −20°C for a three-month period was examined by spiking drug free plasma with known concentrations of tibenelast and assaying them three months later. The determined concentrations found after storage at −20°C for three months showed no loss of potency. The benchtop stability of tibenelast was evaluated at 100 and 1000 ng/ml. Drug-free plasma was spiked with these concentrations and aliquots ($n=3$) were drawn from the samples at 0, 1, 3, 6, 24 and 48 h. These samples were assayed and the results indicated no decay of tibenelast over a 48-h period when stored at room temperature. Freeze–thaw stability was evaluated at the 75 ng/ml concentration. A 75 ng/ml control sample was prepared fresh on day 1 and the concentration of the sample was determined five times. The sample was frozen overnight, thawed, aliquoted and assayed ($n=5$) on the next day. This procedure was repeated on an additional day. The mean of the quintuplet determinations was essentially unchanged over the course of the freeze–thaw cycles (day 1, 70.44 ng/ml; day 2, 70.88 ng/ml; day 3, 71.92 ng/ml). The stability of tibenelast in a processed sample was determined by preparing a batch of samples and allowing them to sit for a 24-h period before analyzing them on the HPLC system. The chromatograms were indistinguishable from the other runs. The results of the analysis gave expected values for the slope, intercept, C.V. and R.S.D. of the standard curve, and the control samples were all within acceptable limits of the analysis as defined by the assay validation.

This procedure has been used to provide pharmacokinetic data in healthy male volunteers following single and multiple doses of tibenelast. Less than 3% of the 100-mg single oral dose of tibenelast appeared in the urine as tibenelast. Fig. 4 shows a tibenelast plasma concentration versus time profile for a volunteer who received a single 100-mg oral dose of tibenelast. This method is intended to support all clinical trials of tibenelast.

CONCLUSION

A sensitive and selective HPLC method was developed for the determination of tibenelast in plasma and urine. The assay was shown to be accurate, precise, sensitive and linear in plasma and urine. Stability of tibenelast in plasma has

been shown under the normally expected conditions of a clinical trial. The assay has been successfully applied to phase I clinical trials.

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