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Column-switching high-performance liquid chromatographic method for the determination of SK&F 106203 in human plasma after fluorescence derivatization with 9-anthryldiazomethane

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ABSTRACT

A sensitive and selective high-performance liquid chromatographic method was developed for the determination of SK&F 106203 3-(2-carboxyethylthio)-3-[2-(8-phenyloctyl)phenyl]propanoic acid, a potent peptidoleukotriene end organ receptor antagonist, in human plasma. The method involves isolation of SK&F 106203 and the internal standard (SK&F 104736) from plasma samples by liquid–liquid extraction prior to derivatization with 9-anthryldiazomethane. The derivatized samples were first subjected to a solid-phase extraction procedure prior to injection onto a short silica column, which is part of a chromatographic system equipped with an automated column-switching device. Column switching was used to heart-cut the chromatographic zone containing the peaks of interest from this first column and transfer it to an analytical silica column for further chromatographic separation. The peaks were quantified with an in-line fluorometer by measuring the fluorescence emission intensity at 415 nm after excitation at 365 nm. An on-column detection limit of 0.625 ng was achieved for SK&F 106203 by optimizing the derivatization and chromatography conditions. The limit of quantification for SK&F 106203, using 250 μl of plasma, was 20 ng/ml. Linear response in SK&F 106203/internal standard peak-height ratios was observed for SK&F 106203 concentrations ranging from 10 to 5000 ng/ml of plasma. Precision and accuracy were within 5% across the calibration range. The assay was sufficiently sensitive, accurate, and precise to support pharmacokinetic studies in humans.

INTRODUCTION

SK&F 106203, 3-(2-carboxyethylthio)-3-[2-(8-phenyloctyl)phenyl]propanoic acid (S, Fig. 1), is a novel peptidoleukotriene end organ receptor antagonist indicated for the treatment of asthma and other pulmonary diseases. The compound has a high affinity for the leukotriene D_4 receptor on lung membranes. This binding prevents and reverses leukotriene-dependent airway contraction and inhibits the leukotriene component of antigen-induced airway contraction [1]. In order to support pharmacokinetic studies, a sensitive method for quantification of S was required. Development of a sensitive high-performance liquid chromatographic (HPLC) method was complicated by the lack of a chromophoric or fluorophoric group in the molecule. Derivatization of the carboxyl groups of S with a suitable fluorophore was considered necessary in order to enhance the detectability of the compound.

Previous reports concerning HPLC analysis of fatty acids and prostaglandins via pre-column fluorescence derivatization with 9-anthryldiazomethane (ADAM) [2–8] have suffered from a lack of routine applicability in measuring these compounds at very low concentrations. These limitations were mainly associated with the relative instability of commer-

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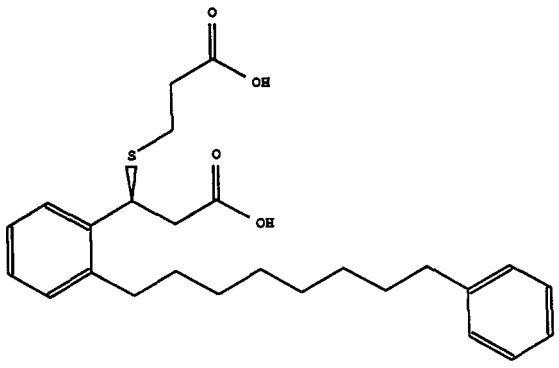
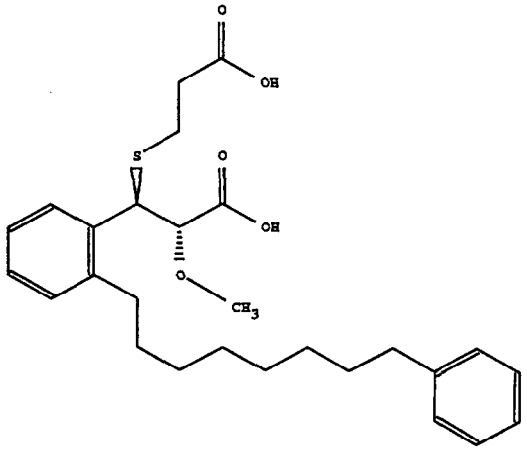


S



IS

Fig. 1. Structures of SK&F 106203 (S) and the internal standard, SK&F 104736 (IS).

cially purchased ADAM, interferences arising from this reagent, and from degradation and by-products formed during the reaction of ADAM with plasma extracts. In the present report, these difficulties were overcome by generating ADAM just prior to use, partially purifying the reaction mixture, and employing automated column switching in order to eliminate various chromatographic interferences.

The method reported here involves isolation of S and the internal standard (SK&F 104736, I.S.) from plasma samples by liquid–liquid extraction prior to derivatization with ADAM prepared *in situ* just prior to use. The derivatized products were then subjected to a solid-phase extraction before injection onto a short silica column, which was part of a chromatographic system equipped with an automated column-switching device. Column switching was used to heart-cut the chromatographic zone containing the peaks of interest from this first column and transfer it to a second analytical column for further chromatographic separation and fluorescence detection.

EXPERIMENTAL

Materials

SK&F 106203 (S) and SK&F 104736 (I.S.) were supplied by Drug Substances and Products, Smith-Kline Beecham Pharmaceuticals (Swedeland, PA, USA). 9-Anthraldehyde, 80% hydrazine hydrate, anhydrous ethyl acetate and quinuclidine were obtained from Aldrich (Milwaukee, WI, USA). N-Chlorosuccinimide was obtained from Fluka (Ronkonkoma, NY, USA). Chloroform was purchased from Polysciences (Warrington, PA, USA). Monobasic and dibasic potassium phosphate, HPLC-grade methanol, hexane, acetonitrile and ethyl acetate were purchased from J. T. Baker (Phillipsburg, NJ, USA). Glacial acetic acid was obtained from Mallinckrodt (Paris, KY, USA). C_{18} solid-phase extraction cartridges (1 ml) and the Vac-Elut SPS 24 manifold were purchased from Analytichem International (Harbor City, CA, USA).

9-Anthraldehyde hydrazone

9-Anthraldehyde hydrazone was synthesized according to a method described previously [9]. An ethanolic solution of 9-anthraldehyde (10 g in 150 ml) was stirred with 80% hydrazine hydrate (10 g) for 3 h at room temperature. The yellow–orange crystals obtained were filtered and dried under vacuum. The compound was stable for three months when stored at −80°C in amber vials.

9-Anthryldiazomethane

ADAM was generated just prior to use by reacting 9-anthraldehyde hydrazone, N-chlorosuccinimide and quinuclidine in ethyl acetate, using a modified method based upon that described by Yoshida *et al.* [3]. To 1 ml of a 13.8 m*M* solution of 9-anthraldehyde hydrazone (3.05 mg/ml of anhydrous

ethyl acetate), 1 ml of 138 m*M* quinuclidine (15.3 mg/ml of anhydrous ethyl acetate) and 1 ml of 13.8 m*M* N-chlorosuccinimide (1.84 mg/ml of anhydrous ethyl acetate) were added and allowed to react for 30 min at room temperature in the dark. The resulting solution was used immediately for derivatization of S and I.S.

Standard solutions and reagents

The stock standard solutions of S and I.S. were prepared by separately dissolving appropriate amounts of each compound in methanol to give a concentration of 1 mg/ml. The stock solution of I.S. was diluted 1:250 with 50% aqueous methanol to give a working standard concentration of 4 μg/ml. Appropriate dilutions of the stock solution of S were made with 50% aqueous methanol to generate a series of working standard solutions (100, 10, 1 and 0.1 μg/ml) which were used in the generation of calibration lines. All stock and working standard solutions were stable for two months when stored at 4°C.

Extraction of SK&F 106203 from plasma

An aliquot of plasma (0.25 ml) containing 75 μl of 50% aqueous methanol (contains standards when preparing calibration curve) and 50 μl of I.S. (4 μg/ml) was mixed with hexane (5 ml) in a 16 × 100 mm screw-cap borosilicate tube. The tube was capped and placed on a linear shaker operating at low speed for 5 min. The tube was then centrifuged for 5 min at 1500 *g* and the hexane layer was aspirated to waste. To the aqueous phase, 5 ml of ethyl acetate – hexane (20:80, v/v) and 0.5 ml of 1 *M* acetic acid were added. The tube was shaken at low speed for 30 min and then centrifuged at 1500 *g* for 5 min. The organic phase was transferred to a clean silanized 16 × 100 mm borosilicate tube and the solvent was evaporated under nitrogen at 40°C. The sample was reconstituted with 100 μl of anhydrous ethyl acetate.

Derivatization with ADAM

To the reconstituted sample from the previous step, 200 μl of freshly prepared ADAM solution were added and allowed to react for 1 h at room temperature in the dark. The reaction mixture was evaporated to dryness under nitrogen at room temperature. The residue was reconstituted with 100 μl of acetonitrile.

Solid-phase extraction of ADAM derivative of SK&F 106203

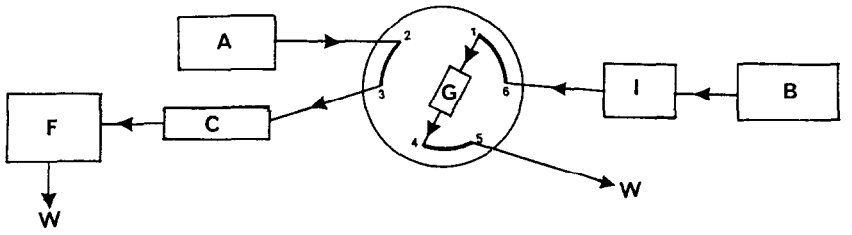
A C_{18} solid-phase extraction cartridge (100 mg/1 ml) was conditioned with 1 ml of methanol and 1 ml of water. The acetonitrile solution, containing the ADAM derivatives of S and I.S., was applied onto the conditioned column and vacuum applied slowly. The column was successively washed with 1 ml of phosphate buffer (0.1 *M*, pH 8.0), 1 ml of water and 3 ml of 85% aqueous acetonitrile. S and I.S. were eluted from the column with 2 ml of acetonitrile. The eluent was evaporated under nitrogen at 40°C. The sample was redissolved first in 10 μl of ethyl acetate and then diluted with 90 μl of hexane. The extract was transferred to a WISP vial and 10–50 μl were injected onto the HPLC system. This partially purified sample was stable for at least 48 h prior to chromatographic analysis.

High-performance liquid chromatography

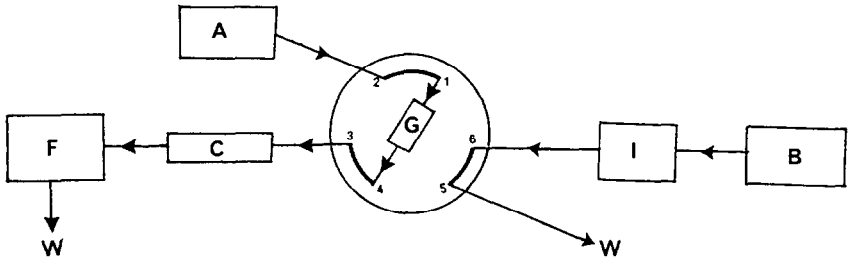
The HPLC system consisted of a column-switching device (Millipore, Milford, MA, USA), two Model 116 isocratic pumps (Beckman, Palo Alto, CA, USA), a Model 710B WISP autosampler (Millipore), and a Model F-1000 fluorescence detector (Hitachi, Danbury, CT, USA). Two columns, a 30 mm × 4.6 mm I.D., 5 μm particle size Spheri-5 silica guard column (Brownlee, San Jose, CA, USA) and a 250 mm × 4.6 mm I.D., 5 μm particle size, Ultrasphere silica analytical column (Beckman) were connected through a column-switching valve as shown in Fig. 2. The analytical column was maintained at 35°C with a temperature controller (Sys-Tec, Minneapolis, MN, USA). The mobile phase consisted of chloroform–ethyl acetate–hexane–glacial acetic acid (2:4:93.8:0.2, v/v) and the flow-rates for the guard and analytical columns were 1 and 1.1 ml/min, respectively. Mobile phase eluents were filtered through a GVWP 0.22 μm filter (Millipore). Detection was accomplished utilizing excitation at 365 nm while monitoring the fluorescence emission at 415 nm. The chromatographic data were collected and analyzed with an automated laboratory system (PE/Nelson Access*Chrom, V 1.6, Cupertino, CA, USA).

Column-switching procedure

The injector (I), two HPLC pumps (A and B), a guard column (G), an analytical column (C), and a



Switch Setting 1 (Off)



Switch Setting 2 (On)

Fig. 2. HPLC system used in this study, showing positions 1 (switch setting off) and 2 (switch setting on). I = Injector; A and B = HPLC pumps; G = guard column; C = analytical column; F = fluorescence detector; W = waste.

fluorescence detector (F) were connected through a column-switching device as shown in Fig. 2. The position of the column-switching device alternated between positions 1 and 2 and was controlled through the timed events option present in the HPLC pump controller. When the column-switching valve was set at position 1 (off position), the solvent from pump B flowed to waste (W) via the injector and guard column, whereas the solvent from pump A flowed through the analytical column and fluorometer to waste. When the valve was switched to position 2 (on position), the solvent from pump B flowed to waste via the injector only and the solvent from pump A flowed through the guard column, analytical column, and fluorimeter. In a typical chromatographic run, the column-switching valve was initially set to position 1 (off) and the sample was injected onto the guard column. The column-switching valve remained in this position for 2.3 min and components which were not retained on the guard column were directed to waste. At the end of this period, the column-switching valve changed to position 2 (on) and remained at this position for 2 min, routing the effluent fraction containing the derivatized products of S and I.S. from the guard column to the analytical column for further chromatographic separation. At the end of the 2-min period (4.3 min post-injection), the column-switching valve was reset to position 1 and remained at this position until the next injection. During this time all the late-eluting components from the guard column were vented to waste. A typical chromatographic run was 40 min in duration.

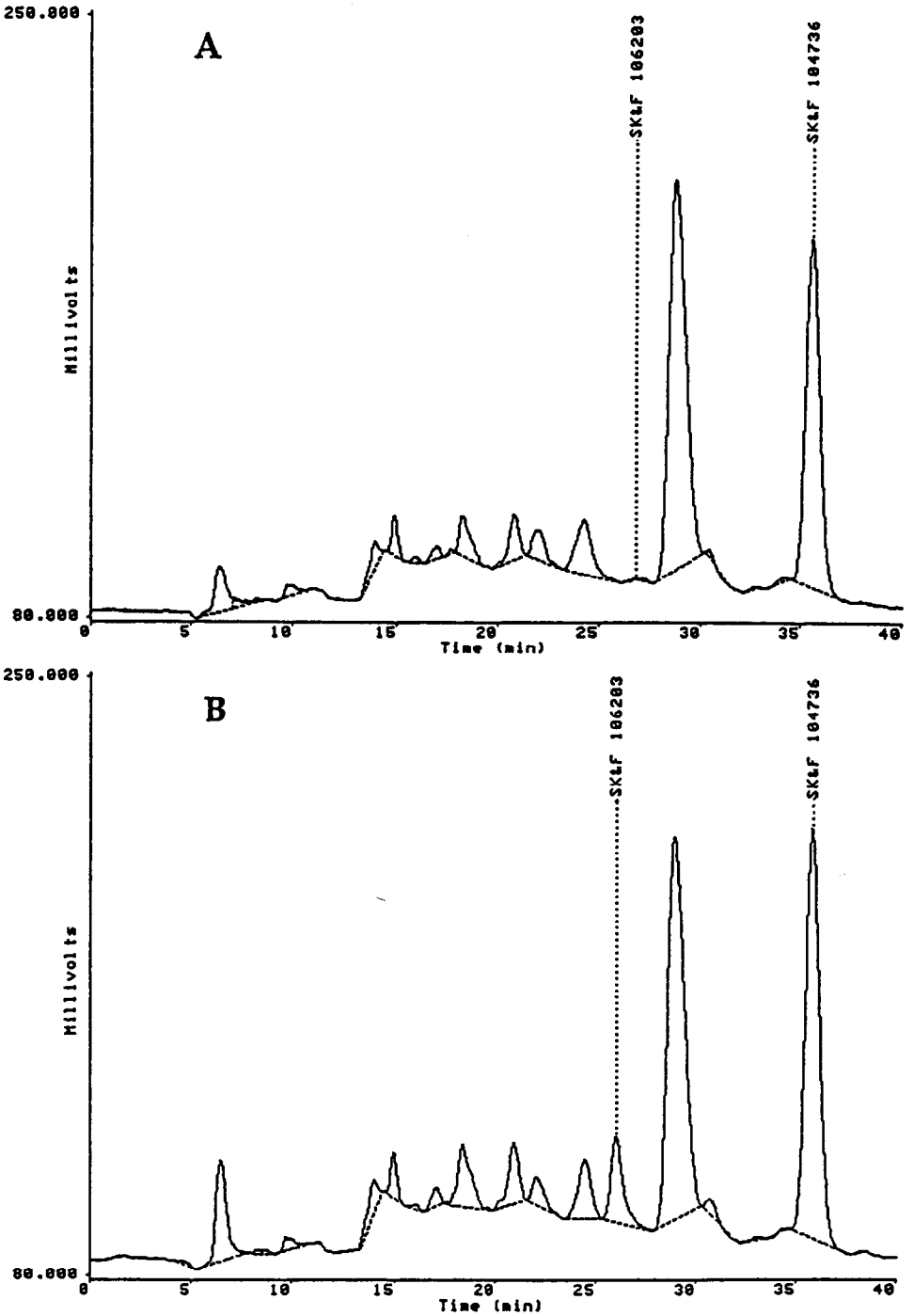


Fig. 3. Chromatograms of (A) drug-free human plasma and (B) plasma to which 100 ng/ml S was added.

TABLE I

ACCURACY AND PRECISION DATA FOR SK&F 106203 IN HUMAN PLASMA

Sample (n = 5)	Day 1	Day 2	Day 3	Intra-day precision[a]	Inter-day precision[b]	Mean accuracy[c]
20 ng/ml				4.44	3.52	103.6
Mean	20.58[d]	20.06	21.50			
S.D.	0.79	0.87	1.11			
C.V. (%)	3.84	4.33	5.16			
Accuracy (%)	102.9	100.3	107.5			
200 ng/ml				2.91	2.25	99.3
Mean	196.97	195.06	203.58			
S.D.	5.92	9.00	2.25			
C.V. (%)	3.01	4.61	1.11			
Accuracy (%)	98.5	97.5	101.8			
2000 ng/ml				1.84	3.18	97.1
Mean	2010.71	1921.98	1892.13			
S.D.	12.00	48.80	45.08			
C.V. (%)	0.60	2.54	2.38			
Accuracy (%)	100.5	96.1	94.6			

[a] Mean of the daily coefficients of variation.
[b] Coefficient of variation of the daily means.
[c] Mean of the daily accuracy calculations.
[d] $n = 3$.

Validation procedure

Three pools of plasma precision samples containing 20, 200, and 2000 ng/ml SK&F 106203 were prepared by adding appropriate volumes of standard solutions to drug-free plasma. Five replicate samples from each pool were extracted and analyzed on three separate days. Concentrations were determined by comparison with a calibration curve prepared on the day of analysis. From the data obtained, intra-day and inter-day precision and mean accuracy were calculated.

TABLE II

BACK-CALCULATED STANDARD CURVE CONCENTRATIONS FOR SK&F 106203

Standard concentration (ng/ml)	Concentration (ng/ml)					C.V. (%)	Accuracy (%)
	Day 1	Day 2	Day 3	Mean ±	S.D.		
10	8.71	7.72	7.10	7.84	0.81	10.4	78.4
20	20.05	21.67	17.41	19.71	2.15	10.9	98.6
30	30.48	33.58	27.56	30.54	3.01	9.9	101.8
50	47.97	50.02	48.90	48.96	1.03	2.1	97.9
100	106.93	114.65	103.60	108.39	5.67	5.2	108.4
200	220.59	210.22	219.13	216.65	5.61	2.6	108.3
500	546.88	497.73	418.27	487.63	64.90	13.3	97.5
1000	1024.36	992.79	974.62	997.26	25.17	2.5	99.7
2000	2025.82	2096.63	2246.72	2123.06	112.80	5.3	106.2
5000	4888.54	4895.57	4894.86	4892.99	3.87	0.1	97.9

RESULTS AND DISCUSSION

Several features of this method were designed to overcome problems encountered with the use of ADAM as a fluorimetric derivatizing agent for carboxylic acids. The instability and decomposition of ADAM during storage, which lead to potential interferences, were circumvented by generating ADAM from the corresponding hydrazone just prior to derivatization. The hydrazone intermediate was stable to storage and was conveniently converted to ADAM via a one-step reaction. Immediate preparation of ADAM not only eliminated the need to store ADAM but also produced reproducible yields of the ester product with fewer interfering degradation products. The reproducibility of the reaction was determined by derivatizing six replicate plasma extracts containing 200 ng/ml each of S and I.S. and measuring the peak heights of the corresponding diester derivatives. The reaction displayed excellent reproducibility, yielding coefficients of variation (C.V.) of 9.0 and 8.6% for S and I.S., respectively. Use of an automated column-switching device also helped to eliminate interfering peaks from the chromatograms, which were related to both reagent impurities and interferences resulting from derivatized endogenous components. Consequently, the overall sensitivity of the method was improved.

Since S was a dicarboxylic acid, reaction with ADAM could possibly yield three fluorescent products, two mono-derivatives and one di-derivative. Chromatographic analysis of the crude reaction mixture, resulting from the reaction of radiolabelled S with ADAM, showed one major fluorescence peak and two very minor peaks related to the drug. Mass spectral analysis of the major fluorescent component indicated that this product corresponded to the di-derivative of S. S was quantified following derivatization with ADAM and chromatographic analysis by monitoring the fluorescent signal resulting from this di-derivative.

The fluorescence HPLC assay described here for the analysis of S involved hexane–ethyl acetate extraction of drug and the I.S. from acidified plasma prior to derivatization with ADAM. Preliminary extraction of plasma with hexane before acidification and final extraction of drug with hexane–ethyl acetate were necessary to remove several neutral lipids that affected both derivatization and chromatography. Solid-phase extraction of S from plasma was also investigated, however, higher recovery of S and I.S. with fewer endogenous peaks was observed with the liquid–liquid extraction method. Direct analysis of plasma extracts, after reaction with ADAM, precluded quantitation of S below 1 μg/ml of plasma due to impurities present in the reagent and interferences resulting from the reaction of ADAM with endogenous components. Solid-phase extraction of the reaction mixture prior to chromatographic analysis eliminated most of the interfering peaks from the chromatogram, but failed to completely eliminate many other minor interfering components. In order to facilitate quantification of S at low nanogram levels, a column-switching system was incorporated. The partially purified derivatized plasma extract was first injected onto a short silica column, which was connected to an analytical column through the column-switching device. After sending the early-eluting interferences to waste, the short column was connected in-line with the analytical column, and derivatized S and I.S. were transferred to the analytical column, for further chromatographic separation.

Sensitivity, linearity, and selectivity

Typical chromatograms of plasma extracts obtained from drug-free human plasma and plasma to which S was added at a concentration of 100 ng/ml are shown in Fig. 3. Endogenous plasma components did not interfere with the quantification of the drug or the I.S. over the concentration range described here. The limit of quantification for S in human plasma was 20 ng/ml, using a 0.25-ml plasma sample. Linear response in the peak-height ratio of S to the I.S. was observed with concentrations of analyte ranging from 10 to 5000 ng/ml. Weighted ($1/y$) linear regression analysis of calibration lines provided the equation $y = 0.001983x + 0.010401$ and a correlation coefficient greater than 0.998.

Accuracy and precision

Results of a three-day validation study are displayed in Table I. At plasma concentrations of S of 20, 200, and 2000 ng/ml, the intra-day precision of the method, as indicated by the mean of the daily C.V.s, was found to be 4.44, 2.91 and 1.84%, respectively. The inter-day precision, as indicated by

the C.V. of the daily means, was 3.52, 2.25, and 3.18% at concentrations of 20, 200, and 2000 ng/ml, respectively. The accuracy of the method, determined from the percent ratio of actual to predicted concentrations, was found to be 103.6, 99.3, and 97.1% at concentrations of S of 20, 200, and 2000 ng/ml, respectively. The precision of the assay was further assessed through back-calculation of the standard curve concentrations. The results are shown in Table II.

Recovery and stability

The recovery of S from plasma was determined using radiolabelled compound, during the initial isolation of drug from plasma by liquid–liquid extraction. The recovery, based on the amount of radioactivity recovered in the organic phase following liquid–liquid extraction, was 84%. The recovery of S and I.S. in the final extract could not be determined due to a lack of authentic standards of the diester derivatives of S and I.S. Plasma samples were stable for at least four months at −80°C. The derivatized extracts were stable for at least 48 h at room temperature.

The quantitative HPLC methodology described here provided for the sensitive and selective determination of S in human plasma, utilizing pre-column derivatization with ADAM and automated column switching. The limit of quantitation using 0.25 ml of plasma was 20 ng/ml. The HPLC method described here has comparable sensitivity to the gas chromatographic–mass spectrometric assay [10], which has been routinely used to quantitate S in various clinical samples, and can be used as an alternative to the instrumentally complex mass spectrometric method.

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