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A specific enzyme immunoassay (EIA) with selective extraction for quantitation of a topical anti-inflammatory agent, SCH 40120, in human plasma

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Abstract: SCH 40120 is a potent anti-inflammatory agent under development for the topical treatment of dermal inflammatory and allergic disorders such as atopic dermatitis, contact dermatitis and psoriasis. We have previously described a sensitive enzyme immunoassay (EIA) for SCH 40120 in unextracted human plasma to support clinical studies. However, severe cross-reaction with unknown metabolites was observed during validation using samples from rats dosed with ^{14}C-SCH 40120. Therefore, a selective extraction procedure was developed to remove the unknown plasma metabolites of SCH 40120 prior to EIA quantitation. The modified EIA using extracted plasma was cross-validated with an LC method using plasma samples from dosed subjects (human and rat), thereby confirming the specificity of the assay. The EIA can reliably quantitate SCH 40120 in plasma samples from 100 pg ml^{-1} to 10 ng ml^{-1} with good linearity, accuracy and precision, and is suitable for pharmacokinetic studies in man.

Keywords: *SCH 40120; enzyme immunoassay; assay validation; 5-lipoxygenase inhibitor; topical anti-inflammatory agent; drug analysis; clinical analysis.*

Introduction

SCH 40120, {10-(3-chlorophenyl)-6,8,9,10-tetrahydrobenzo[b][1,8]naphthyridin-5(7H)-one} (Fig. 1), is a novel potent anti-inflammatory agent that has been shown to be an *in vitro* and *in vivo* inhibitor of the production of leukotrienes [1, 2]. It is a new drug candidate under development for the treatment of dermal inflammatory and allergic disorders such as atopic dermatitis, contact dermatitis and psoriasis. We have previously described a sensitive enzyme immunoassay (EIA) for the determination of SCH 40120 directly in unextracted human plasma, in which a number of structurally related compounds and potential metabolites were tested and little cross-reaction with the antibodies was observed, except a few analogues [3]. However, during evaluation of antibody specificity using samples from rats dosed with ^{14}C-SCH 40120, severe cross-reaction with unknown metabolites was observed. This report describes: (1) evaluation of cross-reaction using *in vivo* rat and human plasma samples; (2) development of a selective extraction procedure to the exclusion of unknown SCH 40120 metabolites in plasma prior to EIA quantitation to ensure the specificity of the assay; (3) validation of the modified EIA using extracted plasma (i.e. linearity, precision and accuracy); and (4) cross-validation with a radiometric LC method to confirm the specificity of the EIA.

Materials and Methods

Materials

All solvents were of LC or GC grade. SCH 40120 and other analogues were obtained from the Schering–Plough Research Institute Compound Distribution Center; ^{14}C-SCH 40120 (specific activity: 7.67 μCi mg^{-1}) was prepared by the Radiochemistry Department, Schering–Plough Research Institute (Kenilworth, NJ, USA). The rabbit anti-SCH 40120 antisera and the horseradish peroxidase (HRP) conjugate (designated SCH 38280-HRP) were prepared as previously described [3]. Bovine serum albumin (BSA), Tween-20, normal rabbit serum and goat anti-rabbit IgG were purchased from the Sigma Chemical Co. (St Louis, MO, USA). The microtiter plate was

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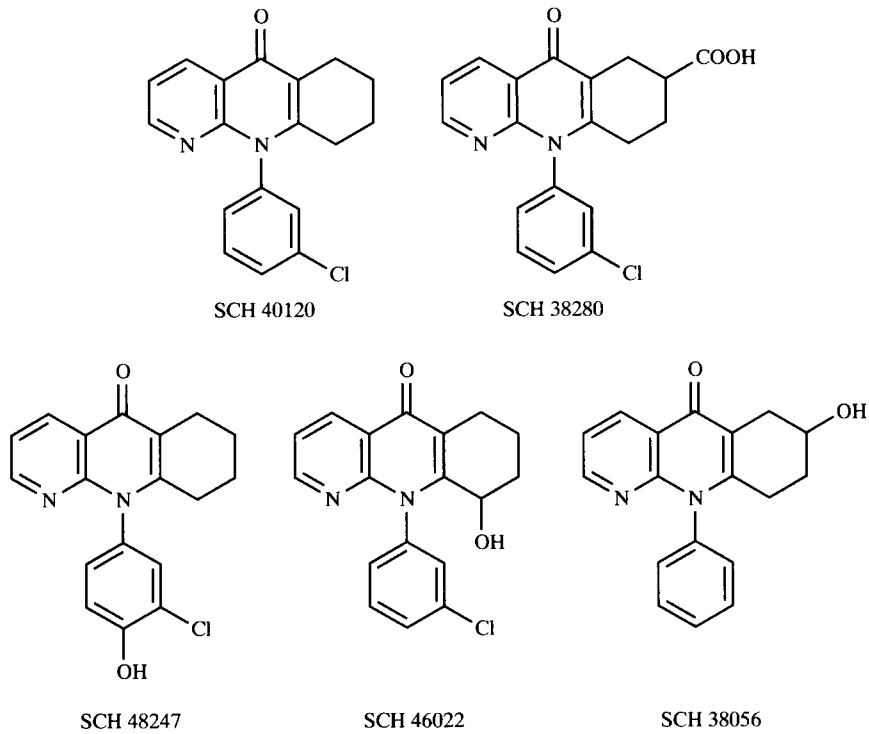

Figure 1
Chemical structures of SCH 40120 and its potential metabolites.

purchased from Nunc, Inc. (Roskild, Denmark. Distributed by Laboratory Disposable Products, Inc., N. Haledon, NJ, USA). The horseradish peroxidase substrate, K-Blue™ (tetramethylbenzidine, TMB), was purchased from Elisa Technologies, Inc. (Lexington, KY, USA). The blank human plasma was obtained from Biological Specialities Co. (Landsdale, PA, USA). The Bio-Tek EL 309 automatic plate reader was purchased from Bio-Tek Instruments, Inc. (Winooski, VT, USA).

Methods

Preparation of standards. The standard stock solution was prepared by dissolving 10 mg of accurately weighed SCH 40120 in 1.0 ml DMF to yield a concentration of 10 mg ml^{-1}. It was diluted in 50% DMF/H_2O to yield a concentration of 100 μg ml^{-1}, which was then further diluted in blank plasma to yield working standards in the concentration range from 50 pg ml^{-1} to 10 ng ml^{-1}. The quality control (QC) samples were prepared from a separate stock solution of SCH 40120 (weighed separately), which was similarly diluted in blank plasma to yield three concentrations (200, 1000, and 5000 pg ml^{-1}) as the low, medium and high QC standards.

Selective extraction procedure. Into a 5-ml plastic tube were added 0.2 ml of plasma (either blank, SCH 40120 standards, other spiked samples, or unknown samples), 0.8 ml of 2.5% DMF in water and 2.0 ml of petroleum ether, then the tube was sealed with a screw-cap and thoroughly mixed. The tubes were shaken on a horizontal shaker for 5 min at 300 rpm and centrifuged at room temperature for 10 min at 3500 rpm. Approximately 1.5 ml of the top layer (petroleum ether) was carefully transferred to another tube while the remaining sample was re-extracted using the same procedure as before. Approximately 1.8 ml of the petroleum ether was again transferred from the second extraction and combined with the first extract. The combined extract was evaporated to dryness using a Savant Speed-Vac® (minimum of 2 h) and the residue was reconstituted in either 0.2 ml of the EIA Buffer (0.1 M phosphate buffered saline, 0.01% Tween-20, 0.2%BSA, pH 7.4) for EIA analysis or 0.1 ml of chloroform and/or methanol for chromatographic (TLC/LC) analysis.

Extraction recovery of SCH 40120. ^{14}C-SCH 40120 (~13,000 dpm) was spiked into 0.2 ml of human plasma (n = 5) and extracted into petroleum ether. The radioactivity was

measured by liquid scintillation counting before and after the selective extraction. A percent recovery of 82% was obtained by comparing the spiked total dpm (n = 3) to the extracted dpm.

Extract analysis by TLC. A human plasma sample spiked with equal amounts (100 μg each) of SCH 40120 (parent drug), SCH 48247 and SCH 46022 (potential metabolites, Fig. 1) was extracted as described and the residue was re-dissolved in chloroform–methanol (1:1). It was applied onto a TLC plate (silica G) and developed in chloroform–methanol (30:1, v/v). The plate was visualized under short-wave UV light with a sensitivity of approximately 1–10 ng/spot.

LC analysis. The LC procedure was used to analyze both *in vivo* spiked plasma and post-dosed *in vivo* plasma samples. The plasma sample (0.5 ml) was mixed with an equal amount of water and applied to a C_{18} Sep-Pak® solid phase extraction (SPE) cartridge which was then washed with water (3 × 1 ml). The organic materials on the column were then eluted with 3 ml of methanol, a 0.1-ml aliquot of which was counted by liquid scintillation counting to determine overall recovery (~90%) from the cartridge. The sample eluent was then evaporated to dryness, re-dissolved in 0.15 ml of methanol, and injected into a Waters C_{18} Novapak® LC Column (3.9 × 150 mm). The column was eluted with acetonitrile/ammonium acetate (0.2 M, pH 6.0) using a gradient (5–35% acetonitrile and 95–65% ammonium acetate) at a flow rate of 1 ml min^{-1}. An UV detector (Waters 490E Model) was used to monitor the spiked samples at 254 nm; eluting fractions were collected at 1 min intervals and the radioactivity quantitated by liquid scintillation counting for radioactive *in vivo* plasma samples.

The EIA procedure and data analysis. The EIA procedure and data analysis method are essentially the same as previously described [3], except the second antibody (anti-rabbit IgG) was diluted 50 fold in the coating buffer (0.1 M Na_2CO_3, pH 9.6).

Table 1
Mean SCH 40120 concentrations in rat plasma (^{14}C-SCH 40120, 10 mg kg^{-1}, single oral dose)

Time (h)	A (by EIA) (ng ml^{-1})	B (radioactivity) (ng equiv./ml)	A/B (%)
1	630	1160	54.3
2	550	910	60.4
4	1060	2110	50.2
6	1390	2630	52.9

Results

Evaluation of the cross-reaction using in vivo *rat plasma samples*

The cross-reactivity of the antisera with a number of structurally related compounds and potential metabolites of SCH 40120 has been examined using an *in vitro* competition assay as previously described [3]. The antisera appeared to be specific to SCH 40120 since little cross-reaction was observed for most compounds tested, except for SCH 38056 (Fig. 1) which mimics the structure of the hapten (SCH 38280, Fig. 1) used for developing the antibodies (~20%). This indicated the potential for severe cross-reaction if this type of metabolite exists *in vivo*. Accordingly, *in vivo* plasma samples from rats dosed with ^{14}C-SCH 40120 (10 mg kg^{-1} single oral administration) were used for validation. It was noted that the ratio of SCH 40120 measured directly in unextracted plasma by EIA to ^{14}C-SCH 40120-derived total radioactivity was constant (~0.5) from 1 to 6 h post dose (Table 1), suggesting that the extent of metabolism did not change with time (which is highly unlikely). However, LC analysis demonstrated that, at 2 h post-dose, only ~10% of the total radioactivity in the unextracted sample was due to the parent drug. This discrepancy indicated that

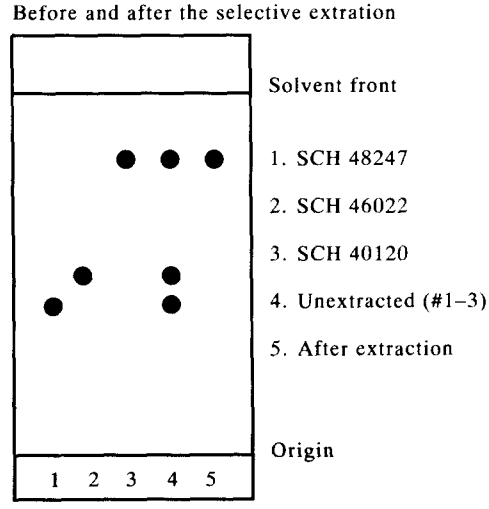

Figure 2
Thin layer chromatogram (TLC) of spiked plasma samples.

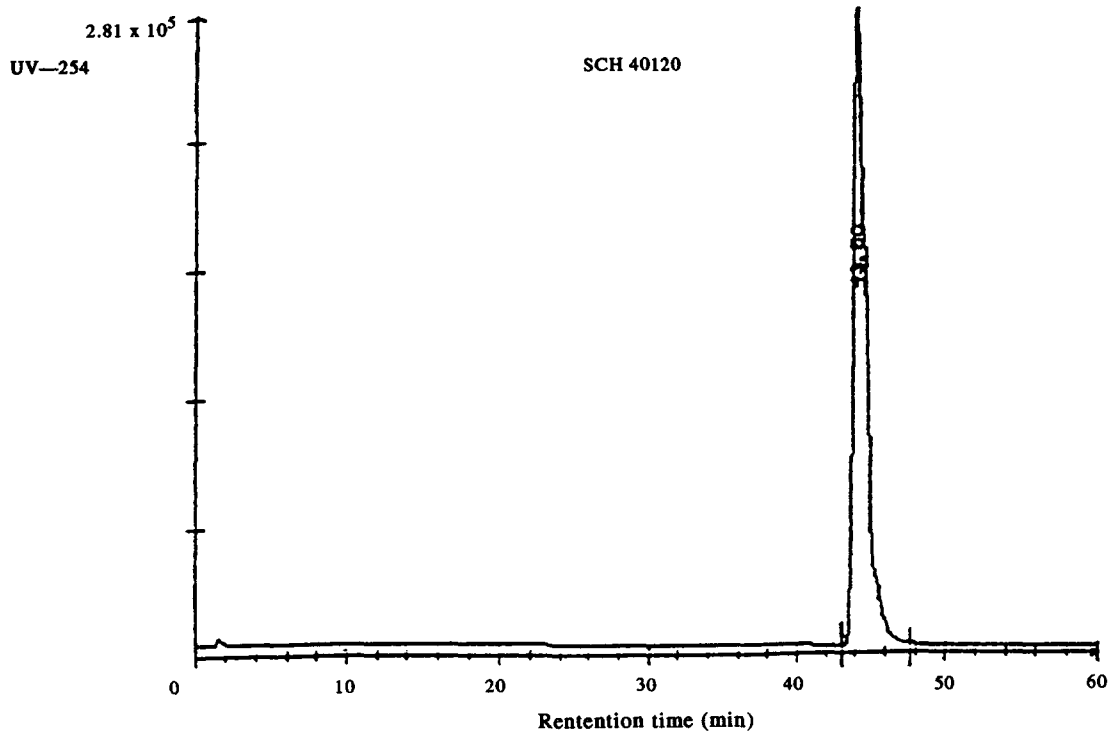

Figure 3
LC profile of a spiked plasma sample post-extraction.

unknown metabolites in rat plasma were cross-reacting significantly with the antibodies. Further LC metabolite profiling studies suggested the presence of SCH 38056-like metabolites in plasma. Therefore, a selective extraction procedure was sought which would remove the polar plasma metabolites of SCH 40120.

Selective extraction

Initially, a human plasma sample spiked with SCH 40120 and its potential metabolites, SCH 48247 and SCH 46022 (Fig. 1), was used as a model for evaluation. Several organic solvents and combinations of varying polarity were tested and the extracts were analysed by TLC. It was determined that SCH 40120 was extracted into petroleum ether to the exclusion of these two potential metabolites (Fig. 2). LC analysis of the same sample confirmed this finding (Fig. 3).

The extraction recovery of SCH 40120 into petroleum ether was determined using plasma samples spiked with ^{14}C-SCH 40120. By comparing the extracted radioactivity ($n = 5$) to the total radioactivity added ($n = 3$), a recovery of 82% was obtained using two extractions per sample.

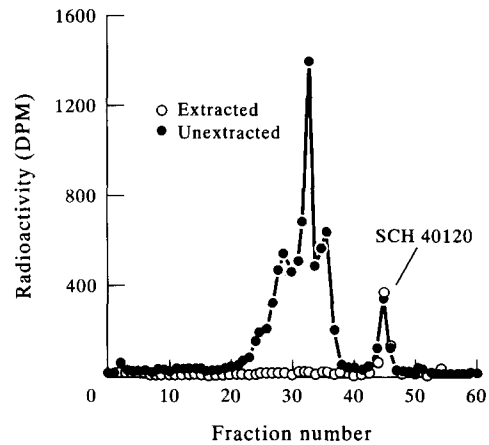

Figure 4
LC profiles of extracted and unextracted rat plasma samples (2 h after single oral dose of 10 mg ^{14}C-SCH 40120/kg).

The validity of this selective extraction procedure was evaluated by analysing a pooled *in vivo* plasma sample from dosed rats (^{14}C-SCH 40120, 10 mg kg^{-1}, single oral administration, 2 h post dose) using the LC method before and after extraction. The radiochromatogram indicated that the majority of the radioactivity in the sample was due to unknown polar metabolites of ^{14}C-SCH 40120 (Fig. 4). These peaks, however, were absent in the chromato-

gram after extraction, indicating that nearly complete separation of SCH 40120 from its polar metabolites had been achieved.

Validation of the modified EIA

The modified EIA using extracted plasma samples was validated according to the general validation guideline [4]. A typical standard curve between 50 pg ml^{-1} and 10 ng ml^{-1} in human plasma is shown in Fig. 5; the standard curve parameters are summarized in Table 2.

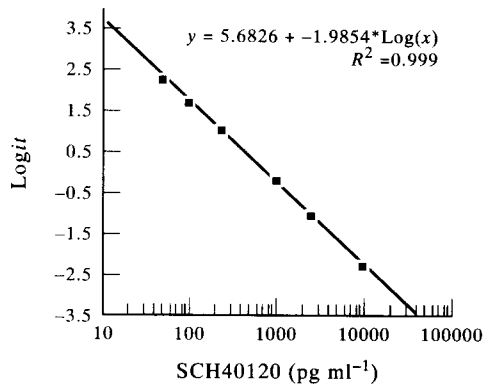

Figure 5
A representative standard curve of the modified EIA for SCH 40120 using a selective extraction procedure.

Table 2
Standard curve parameters

Standard curve	Slope	*y*-intercept	r^2
1	−1.973	5.641	0.999
2	−1.946	5.464	0.998
3	−1.956	4.272	0.998
4	−1.985	5.683	0.999
5	−1.954	4.271	0.998
Mean	−1.963	5.066	0.998
RSD (%)	0.81	14.41	0.05

The correlation of determination (r^2) was 0.998 indicating excellent linearity, while the relative standard deviation (RSD) was 0.81% for the slope, 14.4% for the *y*-intercept and only 0.05% for r^2, demonstrating that the standard curve was highly reproducible. The back-calculated standard concentrations also agreed with the spiked (theoretical) values with a RSD (precision) of 10.3% or less and percentage bias (accuracy) of less than 5.0% (Table 3). Quality control (QC) samples at three concentrations (200, 1000 and 5000 pg ml^{-1}) were analysed on different days; the inter-assay RSD ranged from 6.2 to 10.6%, and the percentage bias ranged from −2.2 to 4.0% (Table 4) demonstrating good precision and accuracy of the assay. The limit of quantitation (LOQ) was established at 100 pg ml^{-1} as described previously [3].

Cross-validation with an LC method

The modified EIA was further cross-validated with an LC method for the quantitation of ^{14}C-SCH 40120 in the *in vivo* samples. The same pooled rat plasma sample mentioned previously was analysed by both EIA and LC. It was found that only 10.7% of the total radioactivity in the sample was due to ^{14}C-SCH 40120; and the concentration determined was 108.9 ng ml^{-1} by LC (based upon the specific activity of the ^{14}C-SCH 40120) and 102.9 ng ml^{-1} by EIA (Fig. 6). A pooled ^{14}C-SCH 40120-containing human plasma sample from a clinical radiolabelled study was also analysed by both EIA and LC. In this sample, only 31% of the total radioactivity was due to unchanged SCH 40120; the concentration determined was 1.71 ng ml^{-1} by LC and 1.55 ng ml^{-1} by EIA (Fig. 7). These results demonstrate that the extraction procedure was selective for SCH

Table 3
Back-calculated concentrations of SCH 40120 standards

	SCH 40120 (pg ml^{-1})					
Standard curve	50*	100	250	1000	2500	10000
1	55.1	98.8	227	944	2559	10479
2	53.8	92.4	264	972	2683	10194
3	45.0	99.7	276	1066	2598	9097
4	54.3	99.4	230	950	2557	10403
5	44.9	99.6	277	1068	2610	9054
Mean	50.6	98.0	255	980	2601	9845
RSD (%)	10.3	3.2	9.6	8.7	2.0	7.2
% Bias	1.2	−2.0	1.9	2.0	4.1	−1.5

*Spiked (theoretical) concentration.

Table 4
Determined concentrations of quality control samples

	SCH 40120 (pg ml^{-1})		
Standard curve	200*	1000	5000
1	193	1087	4866
2	229	1077	5672
3	185	973	4509
4	206	1097	5047
5	188	967	4355
Mean	200	1040	4890
RSD (%)	9.0	6.2	10.6
% Bias	0.0	4.0	−2.2

*Spiked (theoretical) concentration.

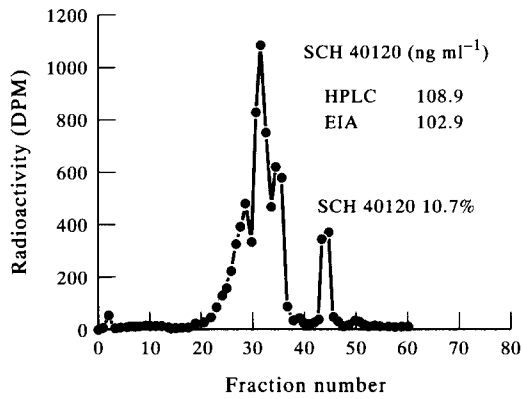

Figure 6
Cross-validation of the modified EIA with an LC method using a pooled rat plasma sample (2 h after a single oral dose of 10 mg ^{14}C-SCH 40120/kg).

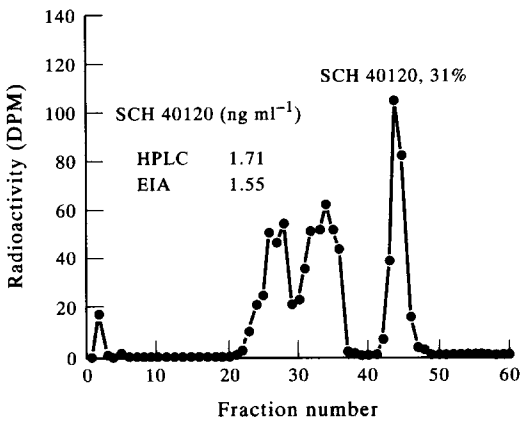

Figure 7
Cross-validation of the modified EIA with an LC method using a pooled human plasma sample from a clinical radiolabelled study.

40120 to the exclusion of its polar metabolites in plasma samples before EIA quantitation. Therefore, the modified EIA with the extraction is specific for SCH 40120.

Discussion

Analytical methods used for the determination of drugs or drug metabolites in biological samples play a significant role in new drug discovery and development. It is essential to use well-characterized and fully validated method for bioavailability, bioequivalence, pharmacokinetic and toxicokinetic studies. A published conference report [4] provided a general guideline on what is required for validation. We have demonstrated our approach for the validation of enzyme immunoassays suitable for pharmacokinetic and toxicokinetic studies [5]. Understanding the inherent limitations of immunoassay technology is important for validation and proper use of this powerful technique. Of particular importance are limitations due to assay interference caused by endogenous or exogenous molecules with structures similar to the principal analyte of interest (i.e. cross-reacting molecules). Although the original EIA for SCH 40120 using unextracted plasma had little cross-reaction with a number of potential metabolites in the *in vitro* binding assay, it became obvious that it suffered severe cross-reaction with unknown metabolites when *in vivo* plasma samples were used for validation. This example demonstrated that the common practice of using *in vitro* binding assays for the evaluation of antibody specificity is insufficient for assay validation. The selective extraction procedure described herein ensured the specificity of the assay for the parent drug since the polar metabolites were excluded prior to EIA quantitation. The specificity was further confirmed by cross-validation with an LC assay using plasma samples from dosed subjects (human and rat). This modified EIA was validated by evaluating its standard curve parameters and the goodness of fit, precision and accuracy of the back-calculated standard concentrations, and the inter-assay precision and accuracy using QC samples at low, medium and high concentrations.

Conclusions

A competitive enzyme immunoassay (EIA) using a selective extraction procedure was developed and validated for the determination of SCH 40120 in human plasma. The method is specific, linear, sensitive, precise and accurate with a limit of quantitation (LOQ) of 100 pg

SCH 40120 per ml of plasma. The assay is suitable for evaluation of the pharmacokinetics of SCH 40120 in man.

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