Journal of Pharmaceutical & Biomedical Analysis
Vol. 10, No. 7, pp. 473–479, 1992
Printed in Great Britain

0731–7085/92 $5.00 + 0.00

A competitive enzyme immunoassay for the direct determination of mometasone furoate (SCH 32088) in human plasma

CHANG-JIN WANG,* ZUGUANG TIAN, KEVIN BYRNES and CHIN-CHUNG LIN

Department of Drug Metabolism and Pharmacokinetics, Schering–Plough Research Institute, Bloomfield, NJ 07003, USA

Abstract: Mometasone furoate (SCH 32088) is a synthetic corticosteroid which has a topical anti-inflammatory activity with a minimal potential for suppressing hypothalamic–pituitary–adrenocortical (HPA) axis. A sensitive competitive enzyme immunoassay (EIA) for measuring SCH 32088 in unextracted human plasma has been developed. The 3-(*O*-carboxymethyl)oxime (CMO) of SCH 32088 was synthesized and conjugated with bovine thyroglobulin, and the product was used for the production of antibodies in rabbits. SCH 32088-3-CMO was also conjugated with horseradish peroxidase, which was used as the tracer. The EIA thus developed can detect 1 pg SCH 32088 per assay or 25 pg per ml of human plasma. It can reliably quantitate SCH 32088 in human plasma from 50 pg ml^{-1} to 2.5 ng ml^{-1} with good linearity, accuracy and precision. The assay can be extended to measure SCH 32088 in plasma of laboratory animals. The availability of this sensitive assay makes it possible to evaluate the pharmacokinetics and toxicokinetics of SCH 32088 in laboratory animals and man.

Keywords: *Enzyme immunoassay; mometasone furoate; anti-inflammatory steroid; drug analysis; clinical analysis.*

Introduction

Mometasone furoate (SCH 32088) is a synthetic corticosteroid which has topical anti-inflammatory activity, while exhibiting a minimal potential for suppressing hypothalamic–pituitary–adrenocortical (HPA) function [1, 2]. SCH 32088 is a promising new drug candidate by oral and nasal inhalation for the treatment of asthma and allergic rhinitis. Although SCH 32088 has exhibited promising biological and pharmacological activities, its metabolism, pharmacokinetics and toxicokinetics have not been evaluated due to the lack of a sufficiently sensitive and reproducible analytical method required by the very low dose regimen of the drug. Based on studies with radiolabelled material, SCH 32088 appears to be widely distributed in various tissues and extensively metabolized following intraperitoneal administration to male rats (unpublished data). Thus the concentrations of the parent drug in plasma are presumed to be in the pg ml^{-1} range and cannot be determined by conventional chromatographic methods. Development of a highly sensitive, specific and reproducible immunoassay for this drug was highly desirable. In this paper, the synthesis of a SCH 32088-3-(*O*-carboxymethyl) oxime (CMO) and its conjugation to bovine thyroglobulin; the production of specific antibodies in rabbit; the conjugation of SCH 32088-3-CMO to horseradish peroxidase (HRP); and the development and validation of a direct enzyme immunoassay (EIA) in human plasma for SCH 32088 are reported.

Materials and Methods

Materials

All the solvents were HPLC or GC grade. Carboxymethoxylamine hemihydrochloride, iso-butyl chloroformate and tributylamine were purchased from Aldrich (Milwaukee, WI, USA). Bovine thyroglobulin, bovine serum albumin (BSA), horseradish peroxidase (EIA grade), Tween-20, complete and incomplete Freund adjuvant, normal rabbit serum and antirabbit IgG were obtained from Sigma (St Louis, MO, USA). The EIA microtiter plate was purchased from Nunc-Immunols (Kamstrup, DK 4000). The horseradish peroxidase substrate, tetramethylbenzidine (TMB), was

* Author to whom correspondence should be addressed.

purchased from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD, USA).

Methods

Synthesis of SCH 32088-3-(O-carboxymethyl) oxime (I). To 15 ml of methanol, 210 mg (0.4 mmol) of SCH 32088 (Batch no. 23047-131), 88 mg (0.4 mmol) of carboxymethoxylamine hemihydrochloride and 40 mg (0.48 mmol) of sodium acetate were added, mixed and refluxed overnight. After cooling to room temperature, the reaction mixture was concentrated and partitioned between ethyl acetate and dilute hydrochloric acid. The ethyl acetate layer was washed with water and dried over anhydrous sodium sulphate. After evaporation of the solvent, the residue was purified by preparative TLC using chloroform–methanol (5:1, v/v) as a solvent system. The product was again purified by preparative TLC using chloroform–methanol (3:1, v/v). The final product was obtained as a white solid with a yield of 75%.

A FAB mass spectrum of SCH 32088-3-CMO (Fig. 1) was obtained on a Finnigan Mat TSQ70 mass spectrometer. The sample was ionized in a glycerol matrix, on a flat copper tip by bombarding the matrix with xenon atoms at an energy of 6 KeV. The assignment for major ions in the spectrum was as follows (*m/e*): 594[M + H]$^+$, 559[M + H − HCl]$^+$, 520[M + H − OCH_2COOH]$^+$, 483[M + H − Furoate]$^+$. The chemical shifts (in ppm) from one-dimensional ^{1}H-NMR (Gemini 300 MHz, DMSO-d_6) were: 8.05(S, 1H, C-27-H), 7.20(d, 1H, C-25-H), 6.70(d, 1H, C-26-H), 6.90(d, 0.5H, C-2b-H), 6.55(dd, 1H, C-1b-H, C-4a-H), 6.35(d, 0.5H, C-1a-H), 6.15(d, 0.5H, C-2a-H), 5.85(S, 0.5H, C-4b-H), 4.50(dd, 2H, C-28-H), 4.30(S, 1H, C-11-H), 4.20(S, 1H, C-21-H). The two-dimensional ^{1}H-NMR spectrum, obtained on a Varian XL400 spectrometer clearly demonstrated that the protons on ring A were evenly divided into two groups and each of them was exactly half of the one proton-equivalent (Fig. 2). The NMR spectrum also demonstrated the coupling relationships among protons on ring A and the furan ring. Therefore, the peak assignment could be deduced and the structure of the final product was confirmed as a 50:50 mixture of the *cis*- and *trans*-oxime isomers of SCH 32088-3-CMO.

Preparation of SCH 32088-3-CMO-thyroglobulin (II). The procedure is based on the mixed anhydride method [3, 4] with some modification (Scheme 1). The SCH 32088-3-CMO (I) (0.5 mg) was dissolved in a mixture containing 100 µl of anhydrous dimethyl formamide (DMF) and 5 µl of tributylamine. Then 5 µl of iso-butylchloroformate was added and the mixture was allowed to stand at room temperature for 15 min. This active labelling reagent ('mixed anhydride') was added dropwise to a solution of 1.5 ml of 50 mM $NaHCO_3$, pH 8.5 and 0.4 ml of DMF containing 20 mg of bovine thyroglobulin at room temperature with stirring. The reaction mixture was then stirred for another 20 min at room temperature. Subsequently, a second batch of the 'mixed anhydride' was prepared and added to the stirring mixture and the reaction was allowed to continue for 1 h at

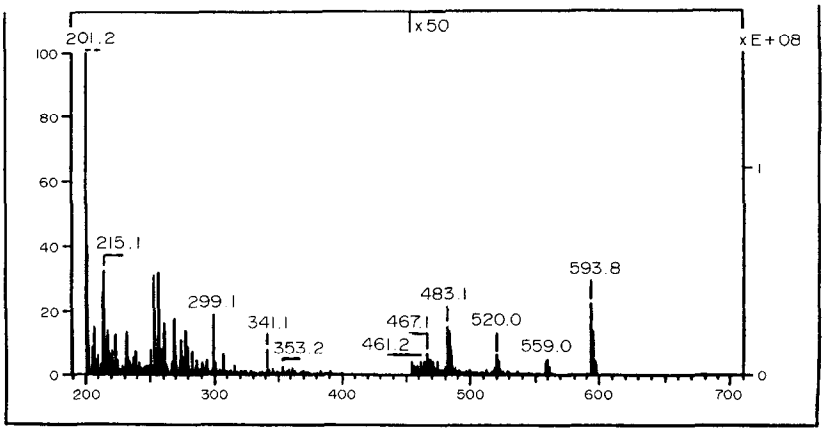


Figure 1
The FAB mass spectrum of the SCH 32088-3-CMO (I). The spectrum was obtained on a Finnigan Mat TSQ70 mass spectrometer.

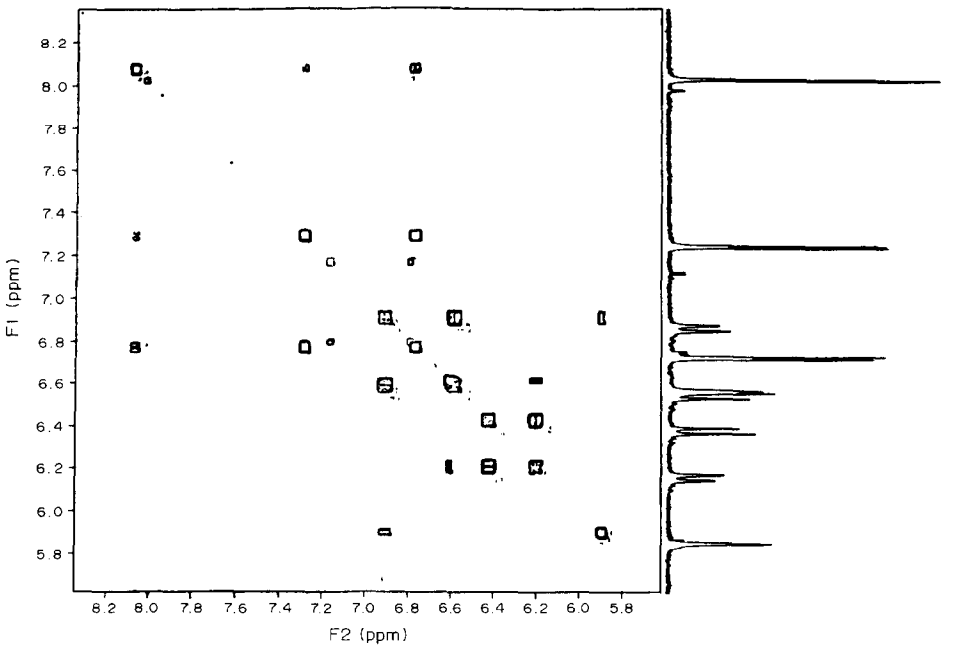
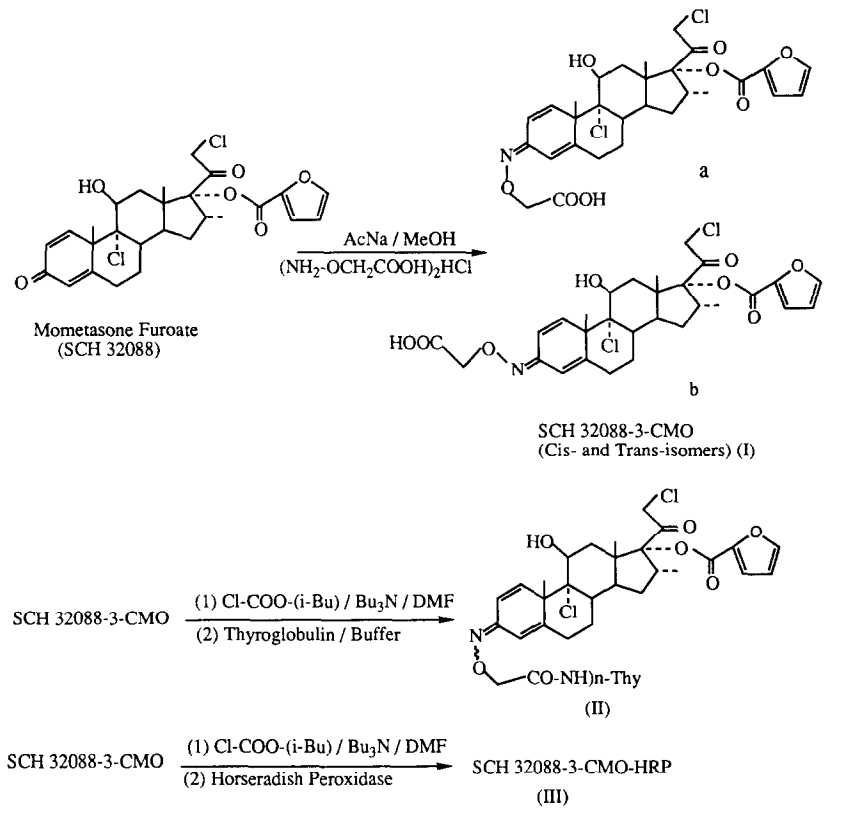


Figure 2
A portion of the two-dimensional ^{1}H-NMR spectrum of SCH 32088-3-CMO (I). It is recorded on a Varian XL400 NMR Spectrometer. See Materials and Methods for details.



Scheme 1

room temperature. This was a 'double labelling' procedure. The reaction mixture was diluted with 2 ml of 0.01 M NaCl and filtered through a cotton pad. The clear solution was then applied onto a Sephadex GF-25 column (25 ml bed volume) and eluted with phosphate-buffered saline (10 mM, pH 7.0) (PBS). The purified product was aliquoted, lyophilized, and stored frozen (−20°C).

Preparation of SCH 32088-3-CMO-HRP. The procedure was essentially the same as above except that the total DMF concentration in the reaction was only 25%. The reaction mixture was purified by two sequential Pierce Desalting Columns and then dialysed against 10 mM phosphate buffer, pH 7.0. The product was aliquoted and stored in the refrigerator (0–4°C).

Immunization schedule. The SCH 32088-3-CMO-Thy (II) (containing 1.0 mg protein) in 0.5 ml of saline was emulsified with 0.5 ml of the complete Freund adjuvant and injected intradermally into the backs of two female New Zealand white rabbits (8–10 weeks old). Booster injections of the same dose with an equal volume of incomplete Freund adjuvant were carried out monthly. The rabbits were bled from an ear vein 7–14 days following each booster injection. The serum was obtained after centrifugation of the blood at 2000*g* for 10 min at room temperature.

The double antibodies EIA procedure. The anti-rabbit IgG was diluted 100 times in 50 mM sodium bicarbonate, pH 8.5 and coated to the plate by incubation of 50 μl (2 μg anti-rabbit IgG) per well at room temperature for 1.5 h. The plate was washed once with the washing buffer (PBS containing 0.05% Tween-20). The diluted normal rabbit serum (NRS, 1 to 20,000 times in the EIA buffer which contained 0.1 M PBS, 0.005% Tween-20 and 0.2% BSA, pH 7.0) was added to the wells in row A of the plate (50 μl per well), and the rabbit antisera with the same dilution as the NRS was added to the rest of the wells (50 μl per well). The plate was incubated at room temperature for 2 h and washed once. The EIA buffer was added to the plate (50 μl per well), followed by blank human plasma, SCH 32088 standard dilution in human plasma, or unknown human plasma samples (50 μl per well) was added. The plate was incubated at room temperature for 1 h. Following addition of 50 μl of the diluted SCH 32088-3-CMO-HRP (1–40,000 times in the EIA buffer) to each well, the plate was incubated for another hour at room temperature and washed three times. The freshly mixed HRP substrate solution was added (0.1 ml per well) and the plate was incubated at room temperature for 15 min. The reaction was terminated by the addition of 0.1 ml of 1 M phosphoric acid. The plate was then read on a Bio-Tek EL 340 plate reader.

Results

Synthesis of SCH 32088-3-CMO

The application of RIA methodology for quantitation of steroids in biological fluids evolves from the elegant work of Erlanger *et al.* who established the immunogenicity of steroids functioning as haptens when conjugated to proteins [3, 4]. The synthesis of intermediate (*O*-carboxymethyl)oxime (CMO) derivative of monocarboxyl steroid involved rather straightforward procedures, however, this is not true for multi-carbonyl systems or a system containing alkaline-sensitive functional groups such as SCH 32088 [5]. Consequently, in order to conjugate SCH 32088 at the C-3 position, a mild and selective procedure was employed for direct conversion of SCH 32088 to SCH 32088-3-CMO (I) with little formation of C-20-CMO, or an epoxide (C-11,9 elimination of HCl) which has been known to occur under alkaline conditions in anhydrous solvents, or by a nucleophilic substition at the C-21 position. As shown in the synthetic scheme, by controlling the molecular ratio of the reactants, the choice of solvents and the use of a mild neutralizing agent, NaAc, a satisfactory yield (75%) was achieved even after two runs of preparative TLC. The structure of (I) was identified by mass spectrometry and proton NMR spectroscopy. The FAB mass spectrum gave not only the correct molecular weight and chlorine isotope pattern, but also several identifiable major fragments of the compound (Fig. 1). The proton NMR spectra indicated that only ring A of the molecule had been derivatized and the product (I) was a 50:50 mixture of the *cis*- and *trans*-oximes at the C-3 position. The complicated relationships among these protons of the two stereoisomers on ring A were resolved by the two-dimensional, 400 MHz proton NMR spectrum as shown in Fig. 2.

Conjugation of SCH 32088-3-CMO to Thy and HRP

SCH 32088-3-CMO (I) was conjugated to bovine thyroglobulin (Thy) and horseradish peroxidase (HRP) via the mixed-anhydride procedure with some modification. Since SCH 32088-3-CMO is very hydrophobic and poorly soluble in 30% DMF in buffer, a 'double labelling' procedure was used to facilitate the conjugation to these proteins. In the reaction with HRP, less DMF was used to protect the enzyme from denaturation.

Immunization and antibody production

SCH 32088-3-CMO-Thy (II) was used to immunize two rabbits, which then produced detectable antibodies to SCH 32088 after the first booster injection. One rabbit (no. 711) produced antibodies with higher titre than the other one, thus the anti-serum was further characterized. SCH 32088-3-CMO-HRP (III) was used to titrate the rabbit antiserum. As shown in Fig. 3, the titration curve clearly demonstrated that the rabbit produced high titre antibodies to SCH 32088. The working titre for the antibodies was 1/20,000. It was also demonstrated that the conjugate (III) exhibited excellent tracer properties in terms of titre (1 to 40,000 dilution) and specific displacement by free SCH 32088 in the assay.

Standard curve and sensitivity

A typical standard curve for SCH 32088 EIA

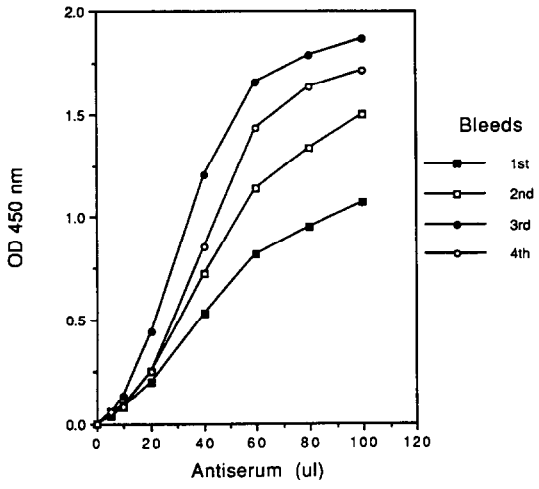


Figure 3
Titration curve of rabbit antiserum with SCH 32088-3-CMO-HRP (III). The antisera from different bleeds were diluted 10,000 times in the EIA buffer, and the SCH 32088-3-CMO-HRP was diluted 20,000 times in the same buffer. See Materials and Methods for experimental details.

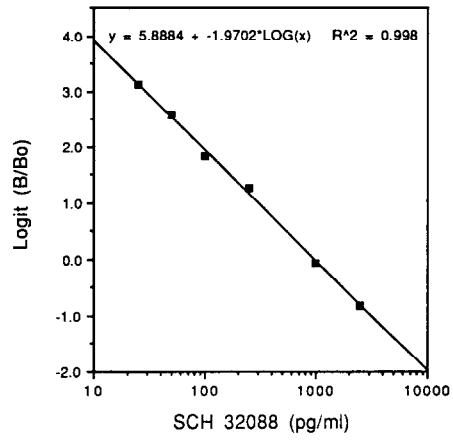


Figure 4
Standard curve of the SCH 32088 EIA in log–logit scale. The SCH 32088 standards were prepared in blank human plasma. The rabbit antiserum was diluted 20,000 times in the EIA buffer, and the SCH 32088-3-CMO-HRP was diluted 40,000 times in the same buffer. For experimental details see Materials and Methods. The logit transformation of the original B/Bo is as follows: $\text{logit}(B/Bo) = \ln[(B/Bo)/(1 - B/Bo)]$.

in human plasma is shown in Fig. 4. In the log–logit plot, the curve was linear over concentrations ranging from 25 to 2500 pg ml^{-1} with a correlation coefficient of 0.997. The limit of detection (LOD), i.e. the lowest concentration that could be differentiated from background ($B/Bo < 0.95$), was estimated at 1 pg $assay^{-1}$ or 25 pg ml^{-1} of spiked concentration in human plasma. The limit of quantitation (LOQ), i.e. the lowest concentration that could be measured with both bias and RSD of less than 20%, was estimated to be 2.5 pg $assay^{-1}$ or 50 pg ml^{-1} of human plasma sample. The measurable range of SCH 32088 was 50–2500 pg ml^{-1} in unextracted human plasma.

Specificity

The cross-reactivity of anti-SCH 32088 antiserum was examined by studying the competitive binding of SCH 32088-3-CMO-HRP with a variety of structurally related steroids, and known or potential metabolites of SCH 32088, endogenous steroidal hormones and common steroidal drugs. As shown in Table 1, insignificant cross-reactivity was observed as measured at 50% displacement of SCH 32088-3-CMO-HRP. The major metabolites, mometasone (hydrolysis of the furoate) and 9-Cl-16-methyl prednisolone (hydrolysis of the C-21-Cl) did not cross-react with the antibodies, even at 1 μg ml^{-1}. Another possible

Table 1
SCH 32088 EIA cross-reaction*

Test substance	% Cross-reaction
SCH 32088 (mometasone furoate)	100
6β-OH mometasone furoate	2.1
9,11-epoxide mometasone furoate	8.1
6β-OH-9α-Cl-16α-methyl PF†	0.08
Mometasone	ND‡
6β-OH mometasone	ND
9,11-epoxide mometasone	ND
9,11-epoxide-21-acetyl PF	ND
9α-Cl-16α-methyl prednisolone	ND
6β-OH-9α-Cl-16α-methyl prednisolone	ND
Androsterone	ND
Oestrone	ND
Progesterone	ND
Hydrocortisone	ND
Norethindrone	ND
17α-Ethynyloestradiol	ND
Dexamethasone	ND

* Assays were run in unextracted spiked human plasma in duplicate.
†PF: prednisolone furoate.
‡ND: not detected.

metabolite, 6β-hydroxy mometasome furoate (MF), cross-reacted minimally. The C-9,11-epoxide MF, which had about 8% cross-reaction with the antiserum, has not been found *in vivo* as a metabolite of SCH 32088. Normal endogenous steroidal hormones and steroidal drugs did not cross-react with the antibodies. Therefore, this antiserum appears to be highly specific for SCH 32088.

Precision and accuracy

The analytical method was evaluated for precision (RSD) and accuracy (bias). As shown in Table 2, the intra-assay was less than 5% for SCH 32088 at concentrations between 50 and 2500 pg ml^{-1}. The intra-assay accuracy was at or less than 5%. The inter-assay RSD and bias were all less than 20%, indicating that the EIA method is reproducible and accurate.

Table 2
SCH 32088 EIA intra-assay precision and accuracy

SCH 32088 (pg ml^{-1})*			
Spiked	Measured	RSD	% Bias
25.0	24.5	1.9	2.1
50.0	49.9	2.2	0.2
100.0	98.6	2.6	1.4
250.0	262.7	3.2	5.1
1000.0	1040.5	2.9	4.0
2500.0	2373.2	3.2	5.1

*The assay was run in unextracted human plasma. Each value is the mean of six replicates.

Application

The application of the EIA method for the analysis of SCH 32088 in clinical samples (Study No. C91-101-01-A, Schering–Plough Corporation) has been conducted. As shown in Fig. 5, the plasma concentration of SCH 32088 in man peaked at about 150 pg ml^{-1} (C_{max}) at 30 min (T_{max}) and then declined rapidly after oral administration of 1 mg of a solution of SCH 32088 to male volunteers. This result clearly demonstrated that this EIA method is suitable for the evaluation of the pharmacokinetics of SCH 32088 in man and possibly in animals after oral administration.

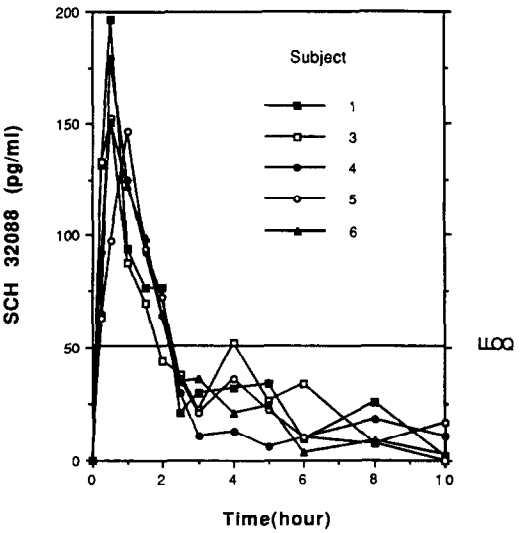


Figure 5
SCH 32088 concentrations in human plasma. A single dose of 1 mg SCH 32088 solution was administered by oral swallowing to each of healthy male volunteers (Study No. C91-101-01-A, Schering Corp.). Blood samples were taken at times indicated and the plasmas were assayed for SCH 32088 concentration. The procedure is described in the Materials and Methods.

Discussion

Radioimmunoassay (RIA) has been widely used for the determination of pg quantities of steroid hormones in blood and other biological fluids [5, 6]. Despite a considerable effort in the past few years, the attempt to develop a sensitive RIA for SCH 32088 has not been successful, mainly due to the complexity of the chemistry involved in the synthesis of a radiolabelled ligand. Several derivatives of SCH 32088 have been synthesized and radioiodinated by several approaches. Unfortunately, the labelled compounds either lost their immunoreactivity or could not be reproduced. Shionogi

Co. attempted to develop an EIA for SCH 32088 by synthesizing SCH 32088-6-0-semi-succinate and conjugating it to BSA. However, the antibodies thus developed suffered a 100% cross-reaction with 6β-hydroxy mometasone furoate, which is one of the major metabolites of SCH 32088.

The EIA described in this report is the first successful approach to quantitate SCH 32088 in human plasma. The LOQ of the assay (limit of quantification, 2.5 pg assay^{-1} or 50 pg ml^{-1} of original concentration of sample), represents one of the highest sensitivities among those RIA and EIA methods for steroids [7–10]. In addition, this analysis is performed directly on plasma samples without prior extraction. Such an advantage is extremely important in the analysis of a large number of samples as in clinical studies. EIA procedures previously employed for steroid determinations have not convincingly demonstrated their suitability for use in direct assays [11–14]. In attempts to improve the sensitivity, reproducibility and ease in performing the method, several approaches, including organic solvent extraction, C18-cartridge isolation, acidifying the human plasma and treating the plasma with Danazol [11, 15, 16] prior to the assay were evaluated. None of these approaches produced better results than the direct assay procedure described herein. Therefore, our assay represents the simplest, most robust yet most sensitive method available for the quantitation of SCH 32088, and appropriate pharmacokinetic and toxicokinetic studies can now be conducted.

Acknowledgements — We would like to thank Dr T.M. Chen for performing the two-dimensional ^{1}H-NMR work; Dr Kwokei Ng for performing the mass spectrometry work; Drs Paul McNamara, Charles Maggatti and Richard Drapper for valuable discussion on chemical synthesis; Dr Mitchell Cayen for his support and guidance on this project; and Ms Barbara Flynn for preparing this manuscript.

References

[1] R.E. Squibb, B.F. Murphy, R.J. Szot, H.E. Black and E. Schwartz, *Eur. J. Pharmacol.* **183**, 2449–2458 (1990).
[2] L. Dominguez, T. Hojyo, E. Vega, M.L. Jones and E. Peets, *Curr. Ther. Res.* **48**, 128–139 (1990).
[3] B.F. Erlanger, F. Borek, S.M. Beiser and S. Lieberman, *J. Biol. Chem.* **228**, 713–727 (1957).
[4] B.F. Erlanger, F. Borek, S.M. Beiser, F. Edel and S. Lieberman, in *Methods in Immunology and Immunochemistry*, Vol. 1, pp. 144–195. Academic Press, New York (1967).
[5] G.D. Niswender and A.R. Midgley, Jr, in *Immunological Methods in Steroid Determination* (F.G. Peron and B.V. Caldwell, Eds), pp. 149. Appleton-Century-Crofts, New York (1970).
[6] H.R. Behrman, *Prog. Clin. Biol. Res.* **285**, 1–14 (1988).
[7] T. Nako, F. Tamamura, N. Tsunoda and K. Kawata, *Steroids* **38**, 11–120 (1981).
[8] H.P. Shah and U.M. Joshi, *J. Steroid Biochem.* **16**, 283–286 (1982).
[9] P. Wood, G. Groom, A. Moore, W. Ratcliffe and C. Selby, *Am. Clin. Biochem.* **22**, 1–24 (1985).
[10] C.J. Munro and B.L. Lasley, *Clin. Biol. Res.* **285**, 289–329 (1988).
[11] R. McGinley and J.R. Casey, *Steroids* **23**, 127–138 (1979).
[12] M. Sauer, J. Foulkes and A. Cookson, *Steroids* **38**, 45–53 (1981).
[13] A. Tuekes, A.O. Turkes, B. Joyce, G. Read and D. Riad-Fahmy, *Steroids* **33**, 347–359 (1979).
[14] B. Joyce, D. Wilson, G. Read and D. Riad-Fahmy, *Clin. Chem.* **24**, 2099–2102 (1978).
[15] W. Ratcliffe, J. Corrie and A. Dalziel, *Clin. Chem.* **28**, 1314–1318 (1982).
[16] W. Ratcliffe, in *Immunoassays for Clinical Chemistry* (W. Hunter and J. Corrie, Eds), pp. 401–409. Churchill Livingston, Edinburgh (1983).

[*Received for review 23 March 1992; revised manuscript received 11 May 1992*]