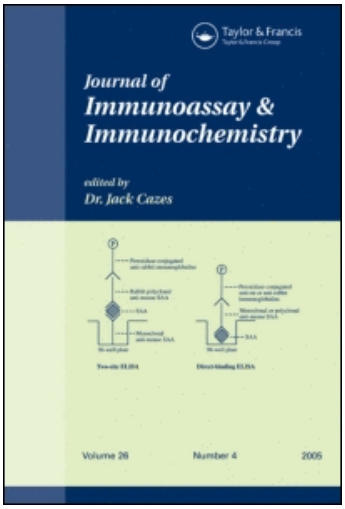
This article was downloaded by:
On: *16 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

An Enzyme Immunoassay for K-76 Monocarboxylic Acid, A Novel Anticomplementary Compound

Itaru Yamamoto[a]; Norio Muto[a]; Kouki Murakami[a]; Sadaharu Suga[a]
[a] Department of Immunochemistry, Faculty of Pharmaceutical Sciences, Okayama University, Okayama, Japan

To cite this Article Yamamoto, Itaru , Muto, Norio , Murakami, Kouki and Suga, Sadaharu(1989) 'An Enzyme Immunoassay for K-76 Monocarboxylic Acid, A Novel Anticomplementary Compound', Journal of Immunoassay and Immunochemistry, 10: 2, 153 — 167
To link to this Article: DOI: 10.1080/01971528908053233
URL: http://dx.doi.org/10.1080/01971528908053233

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

JOURNAL OF IMMUNOASSAY, 10(2&3), 153-167 (1989)

AN ENZYME IMMUNOASSAY FOR K-76 MONOCARBOXYLIC ACID, A NOVEL ANTICOMPLEMENTARY COMPOUND

Itaru Yamamoto, Norio Muto, Kouki Murakami, and Sadaharu Suga
Department of Immunochemistry, Faculty of Pharmaceutical Sciences, Okayama University, 1-1-1 Tsushima-naka
Okayama 700, Japan

Downloaded At: 11:52 16 January 2011

ABSTRACT

A competitive enzyme immunoassay for K-76 monocarboxylic acid (K-76COOH), a novel anticomplementary compound, was developed. K-76COOH was directly coupled with bovine serum albumin through a formation of Schiff base and successive reduction. The spectral data of the conjugate showed no evidence of a Schiff base form. Using the specific antiserum, the proposed homologous assay made it possible to detect K-76COOH at the lowest value of 1 ng/ml of plasma. The immunoassay was validated by the correlation with HPLC analyses. The time courses of plasma levels of K-76COOH after a single oral administration to beagle dogs were precisely determined with a very low absorption efficiency. From these results, it is suggested that the plasma values obtained are insufficient for K-76COOH to exert its anticomplementary action in vivo; thus K-76COOH may have another immunopharmacological function.(KEY WORDS: K-76 monocarboxylic acid, Anticomplementary compound, Enzyme immunoassay, Polystyrene ball, β-D-Galactosidase)

INTRODUCTION

K-76 monocarboxylic acid (K-76COOH), [6,7-dihydroxy-2,5,5,8a-tetramethyl-1,2,3,4,4a,5,6,7,8,8a-decahydronaphthalene-1-spiro-2'-(7'-formyl-6'-carboxyl-4'-hydroxy-2',3'-dihydrobenzofuran)], is a derivative form of K-76 (1) and the structure has been ascertained by its total synthesis (2,3). K-76 was isolated as a new anti-

Copyright © 1989 by Marcel Dekker, Inc.

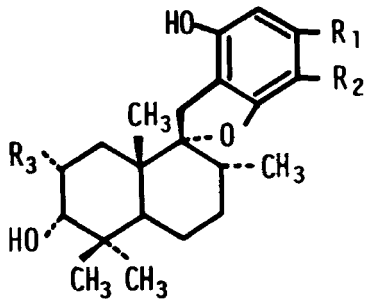
complementary compound from the culture filtrate of Stachybotrys complementi (1,4). K-76COOH was shown to inhibit complement activation in both classical and alternative pathways as well as K-76 (1,5-7). Furthermore, K-76COOH showed less toxicity than K-76. Recently, this compound has been shown to be effective with some immunocomplex diseases, such as glomerulonephritis and systemic lupus erythematosus-like diseases (8). These results are suggestive of other pharmacological effects including antiinflammatory and antiallergic activities. However, its pharmacological mechanism of action and pharmacokinetics have not yet been clarified. For the purpose of elucidating these problems, a sensitive and specific assay method for this compound is required. In this report, we describe the development of a highly sensitive enzyme immunoassay for K-76COOH and its availability for the determination of plasma concentration after an oral administration of this compound to experimental animals. Moreover, we discuss whether or not its anticomplementary action is responsible for some pharmacological effects mentioned above.

Downloaded At: 11:52 16 January 2011

MATERIALS AND METHODS

Materials

K-76, K-76COOH, and some other analogues were generously donated from Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan). Their chemical structures are given in Fig. 1. β-D-Galactosidase (β-Gal, Escherichia coli, grade VIII, 580 units/mg of protein), bovine serum albumin (BSA, fraction V), and 4-methylumbelliferyl-



Compound	R_1	R_2	R_3
K-76	CHO	CHO	OH
K-76COOH	COOH	CHO	OH
K-76CH_2OH	COOH	CH_2OH	OH
K-76$(COOH)_2$	COOH	COOH	OH
K-76OH	COOH	OH	H

FIGURE 1. Structure of K-76COOH and its related compounds.

Downloaded At: 11:52 16 January 2011

β-D-galactoside (4-MUG) were purchased from Sigma Co. (St. Louis, MO). Goat immunoglobulin G (IgG) fraction of anti-rabbit IgG was prepared in this laboratory by ammonium sulfate precipitation and ion-exchange chromatography and used as the second antibody. Freund's complete and incomplete adjuvants were obtained from Difco Labs. (Detroit, MI). Polystyrene balls (1/4 inch in diameter) were purchased from Immunochemical Co. (Okayama, Japan). All other reagents employed were of analytical grade. The following buffers were used: A_1 buffer is 100 mmol/l sodium phosphate buffer (pH 7.4) containing 100 mmol/l NaCl, 1 mmol/l $MgCl_2$, and 1 g/l BSA.

A_2 buffer is 100 mmol/l sodium phosphate buffer (pH 7.4) containing 100 mmol/l NaCl, 1 mmol/l $MgCl_2$, and 10 g/l BSA.

Preparation of Immunogen

K-76COOH was conjugated to BSA through a Schiff base formation between the aldehyde group of the compound and the amino groups of the protein, followed by selective reduction of the bond (9). A typical reaction was as follows: 13.2 mg (30 μmol) of K-76COOH was mixed with 20 mg (300 nmol) of BSA in 100 mmol/l sodium phosphate buffer (pH 8.0) at room temperature for 2 hr and, then, 1.9 mg (30 μmol) of sodium cyanoborohydride was added directly to reduce the Schiff base at room temperature for 92 hr. The reaction mixture was dialyzed against 154 mmol/l NaCl at 4°C for 2 days. The amount of K-76COOH covalently bound to BSA was calculated from the spectral data of the conjugate and by using the values of 3520 and 1830 as molar extinction coefficients at 260 and 295 nm, respectively. These values were based on the reduced form of K-76COOH, in which the aldehyde group was reduced with sodium borohydride. The protein concentration was determined by the method of Lowry et al. (10) with BSA as a standard. Then, the molar ratio of hapten to BSA was determined to be 15.

Immunization

K-76COOH-BSA conjugate was injected subcutaneously in rabbits using 0.5 mg of protein in Freund's complete adjuvant. Booster injections of 0.2 mg of protein in Freund's incomplete adjuvant were given at two-week intervals.

Downloaded At: 11:52 16 January 2011

Downloaded At: 11:52 16 January 2011

Detection of Specific Antibody

The titer and specificity of the antibody produced were determined in an enzyme-linked immunosorbent assay method. A 96-well microtiter plate was coated with K-76COOH-ovalbumin conjugate, which was prepared in the same way as described for the preparation of K-76COOH-BSA conjugate. After blocking with 5 g/l gelatin, each serum sample was applied to a well. Then, peroxidase-labeled goat antibody specific to rabbit IgG was added and enzyme activity trapped on the surface of the well was finally determined by using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) as a substrate. After termination of enzyme reaction, the absorbance at 405 nm was measured in a Sanko microtiter reader.

Preparation of K-76COOH-β-Gal Conjugate

The following manipulations were carried out at 0-4°C. K-76COOH-β-Gal conjugate was synthesized through a Schiff base formation, followed by its mild reduction with sodium cyanoborohydride for 40 hr. The reaction mixture was dialyzed against 154 mmol/l NaCl and its hapten-enzyme conjugate thus obtained was stored at -20°C after mixing the equal volume of glycerol. Enzyme activity was stable for more than 6 months under this condition.

Preparation of Assay Samples

K-76COOH was dissolved in 154 mmol/l NaCl and administered orally to Wistar rats and beagle dogs. Blood samples were taken in heparinized syringes at the indicated times and the plasma

obtained was kept at -20°C until use. Each plasma was once diluted with A_2 buffer and subjected to the assay.

Assay Procedure

Enzyme immunoassay for K-76COOH was performed on the basis of the double antibody solid phase principle. In this assay, K-76COOH-β-Gal and anti-K-76COOH serum were employed after dilution of 1:5000 and 1:10000, respectively, with A_2 buffer. Standard solution of K-76COOH was dissolved in distilled water and diluted to the indicated concentrations with A_2 buffer. The second antibody was immobilized on polystyrene balls by physical adsorption, as described previously (11).

For the assay, 100 µl of standard or sample solution was incubated with 50 µl of diluted β-Gal-labeled K-76COOH and diluted anti-K-76COOH serum at 4°C for 18 hr. Normal plasma was added into each standard tube at the same concentration as that in sample tube. Then, 200 µl of A_2 buffer and a second antibody-bound polystyrene ball were added to each solution and the tubes were agitated at 15°C for 4 hr. The balls were thoroughly washed with A_1 buffer and transfered to another tube containing 200 µl of A_1 buffer. The enzyme activity on the polystyrene ball was determined by incubating with 200 µl of 0.3 mmol/l 4-MUG at 37°C for 2 hr. The reaction was terminated by the addition of 2 ml of 100 mmol/l sodium carbonate and the fluorescence intensity was measured in a Shimadzu fluorospectrometer with the excitation at 370 nm and the emission at 450 nm. B/B_0 value (%) was calculated by the following equation:

Downloaded At: 11:52 16 January 2011

$$B/B_0\ (\%) = \frac{B - Bl}{B_0 - Bl} \times 100$$

B: fluorescence intensity of sample

B_0: fluorescence intensity of zero standard

Bl: fluorescence intensity of blank

RESULTS

Stability of K-76COOH-BSA Conjugate

Intermediate Schiff base showed a characteristic spectrum of K-76COOH after a dialysis against distilled water, whereas such a spectrum disappeared after dialysis against saline. In contrast to this unstable form, the reduced product of the Schiff base gave a new spectrum based on the stable secondary amine compound. This conjugate was used for the immunization in rabbits.

Sensitivity and Specificity of the Proposed Enzyme Immunoassay

High antibody titer in all rabbits was demonstrated after the fourth booster injection and the antiserum with the highest titer was used in this study. A typical standard curve is given in Fig. 2. In this homologous EIA system, K-76COOH was measured in the range of 100—30000 pg. Specificity of the antibody was assessed (Fig. 3) with some chemical derivatives of K-76COOH illustrated in Fig. 1. K-76CH_2OH, which was quantitatively converted from K-76COOH by the reduction with sodium borohydride, showed a higher reactivity to the antibody than K-76COOH, whereas K-76, a dialdehyde form, showed less reactivity. In addition, the dicarboxylic acid derivative was considerably less reactive

Downloaded At: 11:52 16 January 2011

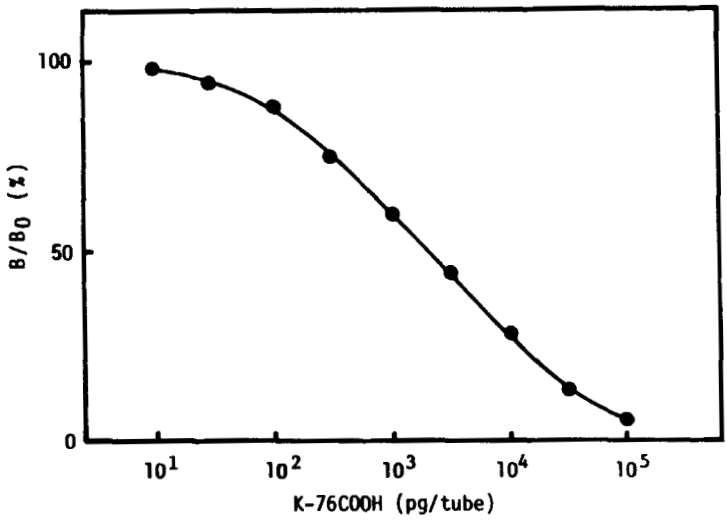


FIGURE 2. Typical standard curve for K-76COOH.

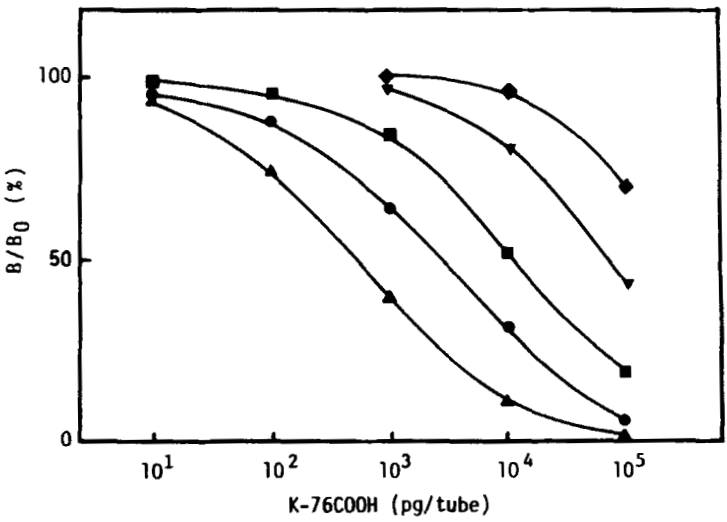


FIGURE 3. Cross-reactivity of some related compounds in the proposed enzyme immunoassay. K-76 (■), K-76CH_2OH (▲), K-76$(COOH)_2$ (▼), and K-76OH (◆) were added in the competitive reaction mixture. Assays were performed as described for K-76COOH (●).

Downloaded At: 11:52 16 January 2011

TABLE 1

Reproducibility of K-76COOH determination by the proposed enzyme immunoassay

	Intra-assay (n=3)			Inter-assay (n=4)		
Sample	M(pg/tube)	S.D.	C.V.	M(pg/tube)	S.D.	C.V.
1	365	22	6.0	381	27	7.1
2	1603	133	8.3	1643	115	7.0
3	1627	155	9.5	1676	92	5.5
4	3700	400	10.8	3754	176	4.7

M: mean of assays
S.D.: standard deviation of mean
C.V.: coefficient of variation (%)

Downloaded At: 11:52 16 January 2011

with the antibody. Thus, the antibody could effectively recognize the structural difference on the benzene ring of K-76COOH.

Precision and Validity of the Enzyme Immunoassay

A dilution test was performed with rat plasma samples, which were serially diluted 1:4 to 1:64 with A_2 buffer. The results showed a good linear relationship between dilution rates and measured values (r=0.999). Table 1 shows intra-assay and inter-assay reproducibility of the proposed EIA for four samples of rat plasma. The precision was good enough to enable the measurement of K-76COOH in plasma, as can be judged from the values of coefficients of variation.

To validate the assay, a range of concentration of K-76COOH in plasma of beagle dogs were analyzed by both EIA and HPLC. EIA showed good correlation with HPLC at higher concentrations (Fig. 4A), and even at much lower concentrations (Fig. 4B).

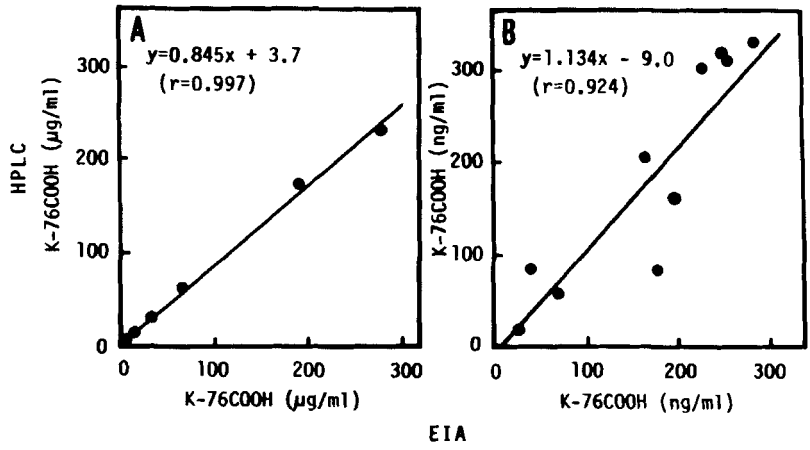


FIGURE 4. Correlation between the enzyme immunoassay and the HPLC method with beagle dog plasma samples. Plasma concentration of K-76COOH was directly determined in the enzyme immunoassay just after dilution with the buffer. In the HPLC method, it was extracted with ethylacetate from acidified plasma and analyzed by using a reverse phase column. The detectable limit of this compound in the latter system was approximately 20 ng/ml of plasma.

Downloaded At: 11:52 16 January 2011

Plasma Levels of K-76COOH in Beagle Dogs

Using this EIA, plasma levels of K-76COOH after a single oral administration were examined in beagle dogs. The time course of plasma levels of K-76COOH is shown in Fig. 5. An oral dose of 1 mg/kg to beagle dogs gave the maximum level around 4 hr with the plasma concentration of 240 ng/ml. Twenty-four hr after administration, the concentration in plasma of beagle dogs remained only about 1/10 of its maximum value. In addition, only this assay gave an exact value when a small amount of K-76COOH (100 μg/kg) was administered; at this level it could not be detected by HPLC.

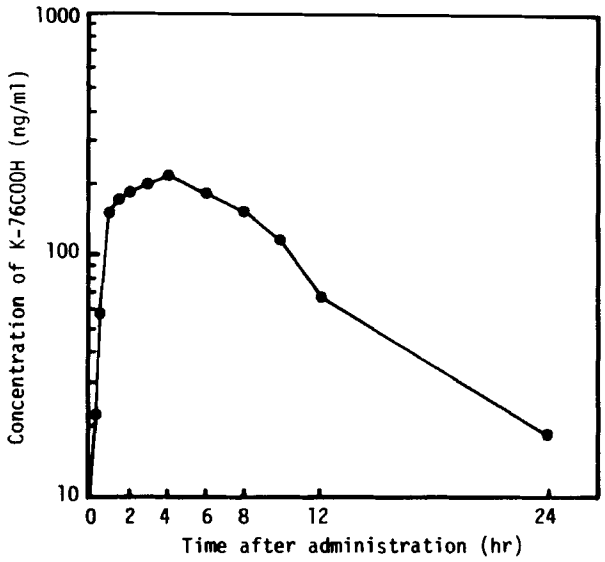


FIGURE 5. Plasma levels of K-76COOH after a single oral administration in beagle dogs. Plasma samples were diluted 1:20 with the buffer and subjected to the assay. Each point represents mean value of two animals.

Downloaded At: 11:52 16 January 2011

DISCUSSION

The antibody specific to K-76COOH was raised in rabbits by immunizing with a K-76COOH-BSA conjugate that was synthesized through a Schiff base formation and successive mild reduction. No antibody was produced in rabbits receiving the Schiff base itself. This indicates that the intermediate Schiff base form easily dissociates into the free components under the physiological conditions. This phenomenon was confirmed by the spectral data after the dialysis of this intermediate against distilled water

and saline. The antibody produced recognized the structure of the coupling site of this hapten more selectively, with the result that the reduced form of K-76COOH strongly replaced K-76COOH in the competitive reaction. However, since the compounds showing cross-reactivity with K-76COOH in this study are all derived chemically and considered not to be produced in vivo, we can practically ignore the interference of these compounds in this determination. In fact, no trace of metabolites of K-76COOH has been detected in plasma of various experimental animals by the HPLC method (Tominaga, N., personal communication). The lack of metabolism of this compound in plasma made the new assay method possible for the determination of its exact concentration.

Downloaded At: 11:52 16 January 2011

The proposed enzyme immunoassay allowed the determination of K-76COOH at levels from 100 to 30000 pg. Plasma samples could be applied to this assay only after dilution with the buffer used. The intra- and inter-assay precision indicated satisfactory assay performance. In addition, this assay was observed to correlate well with the HPLC method when plasma samples from beagle dogs were used. It could be considered that the EIA was superior to the HPLC and radioisotopic assay methods in the sensitivity, the simplicity of sample preparation, and the easiness of the assay procedure. These results indicate that the proposed enzyme immunoassay for K-76COOH is valid and reliable and that it would be available for determination of low levels of human samples. An improvement of the sensitivity of the enzyme immunoassay may be required in subsequent studies. We consider that a heterologous

system will resolve this problem, as described in our previous papers (12,13).

Using the assay described in the present paper, the exact plasma concentration of K-76COOH could be effectively monitored in beagle dogs that received a single oral administration of desirable and biologically relevant dosage. In addition, monkeys showed quite similar absorption profiles, whereas rats showed about 20 times less absorption efficiency than beagle dogs and monkeys (data not shown). Consequently, the absorption efficiencies of this compound were found to be apparently different amoung animal species. This tendency was consistent with the result obtained by the radioisotopic method (Miyamoto, G., personal communication). In this study, maximum plasma concentration was demonstrated to be 240 ng/ml (5.74×10^{-7} mol/l) when K-76COOH (1 mg/kg) was administered to beagle dogs. Miyazaki et al. (1) have reported that K-76COOH causes 50% inhibition of complement-mediated hemolysis at 150 μg/ml (3.41×10^{-4} mol/l). These findings indicate that the plasma concentrations obtained here are too low to exhibit its anticomplementary activity. Judging from these results, some immunopharmacological effects reported previously (8) may result from biological actions different from its anticomplementary action. In fact, we have preliminary data indicating that this compound has some immunomodulating effects on mitogen-induced lymphocyte proliferation and autoantibody production at very low concentrations (manuscript in preparation). These new findings may be responsible for its pharmacological activities that were previously observed in rats that received a low dose of K-76COOH (8).

Downloaded At: 11:52 16 January 2011

To further the elucidation of its pharmacological effects, the pharmacodynamics of this compound should be examined together with the determination of local concentration at the target tissues or active sites. The proposed enzyme immunoassay for K-76COOH would be an effective means to clarify its mechanism of action.

ACKNOWLEDGEMENTS

We would like to express our gratitude to Dr. D. R. Webb of Syntex Research (U.S.A.) for his critical review and help in preparation of this manuscript. We also wish to thank Dr. T. Shimizu and Miss N. Tominaga of Drug Safety Research Center, Otsuka Pharmaceutical Co. Ltd. for providing plasma samples and measuring plasma K-76COOH by HPLC.

Downloaded At: 11:52 16 January 2011

REFERENCES

1. Miyazaki, W., Tamaoka, H., Shinohara, M., et al. A Complement Inhibitor Produced by Stachybotrys complementi, nov. sp. K-76, a New Species of Fungi Imperfecti. Microbiol. Immunol. 1980; 24: 1091-108.

2. Corey, E.J. and Das, J. Total Synthesis of the Complement Inhibitor K-76 in Racemic Form. Structural Assignment to "K-76 Monocarboxylic Acid". J. Am. Chem. Soc. 1982; 104: 5551-3.

3. McMurry, J.E. and Erion, M.D. Stereoselective Total Synthesis of the Complement Inhibitor K-76. J. Am. Chem. Soc. 1985; 107: 2712-20.

4. Kaise, H., Shinohara, M., Miyazaki, W., et al. Structure of K-76, a Complement Inhibitor Produced by Stachybotrys complementi, nov. sp. K-76. J. Chem. Soc. Chem. Commun. 1979; 1979: 726-7.

5. Hong, K., Kinoshita, T., Miyazaki, W., Izawa, T., and Inoue, K. An Anticomplementary agent, K-76 Monocarboxylic Acid: its Site and Mechanism of Inhibition of the Complement Activation Cascade. J. Immunol. 1979; 122: 2418-23.

6. Hong, K., Kinoshita, T., Kitajima, T., and Inoue, K. Inhibitory Effect of K-76 Monocarboxylic Acid, an Anticomplementary Agent, on the C3b Inactivation System. J. Immunol. 1981; 127: 104-8.

7. Hong, K., Kinoshita, T., and Inoue, K. Simple Methods for Preparing EAC1,4b,2a,3b and EAC4b,3b with Human or Guinea pig Complement Components using an Anticomplementary Agent, K-76 Monocarboxylic Acid. J. Immunol. 1981; 127: 109-14.

8. Miyazaki, W., Izawa, T., Nakano, Y., et al. Effects of K-76 Monocarboxylic Acid, an Anticomplementary Agent, on Various in vivo Immunological Reactions and on Experimental Glomerulonephritis. Complement 1984; 1: 134-46.

9. Borch, R.F., Berstein, M.D., and Durst, H.D. The Cyanohydridoborate Anion as a Selective Reducing Agent. J. Am. Chem. Soc. 1971; 93: 2897-904.

10. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem. 1951; 193: 265-75.

11. Yamamoto, I., Tsuji, J., Takai, T., and Fujimoto, M. Double Antibody Enzyme Immunoassay for the Quantitation of Adenosine 3',5'-Cyclic Monophosphate (Cyclic AMP) and Guanosine 3',5'-Cyclic Monophosphate (Cyclic GMP) in Tissues and Plasma. J. Immunoassay 1982; 3: 173-96.

12. Yamamoto, I. and Iwata, K. Enzyme Immunoassay for Clenbuterol, a β_2-Adrenergic Stimulant. J. Immunoassay 1982; 3: 155-71.

13. Yamamoto, I., Matsuura, E., Horiba, M., Akima, K., Nomura, K., and Aizawa, T. Enzyme Immunoassay for Mabuterol, a Selective β_2-Stimulant in the Trachea. J. Immunoassay 1985; 6: 261-76.

Downloaded At: 11:52 16 January 2011