Journal of Pharmaceutical & Biomedical Analysis
Vol. 7, No. 8, pp. 955–963, 1989
Printed in Great Britain

0731–7085/89 $3.00 + 0.00

A radioimmunoassay for physostigmine in biological fluids and tissues

R. L. MILLER* and P. S. VERMA

Department of Internal Medicine, Howard University Hospital, Washington, DC 20060, USA

Abstract: Antibodies were produced in rabbits immunized with physostigmine conjugated to bovine serum albumin (BSA) by two different methods: a diazo immunogen coupled to BSA with carbodiimide and a Mannich coupled immunogen. Both immunogens produced antibodies that could be used to develop sensitive and specific radioimmunoassays (RIA) for physostigmine. The limit of detection of the RIA is 100 pg of physostigmine in plasma or tissue homogenates (1.0 ng ml^{-1}), without the need for an extraction procedure. The interassay and intraassay coefficients of variation were <13% in the RIA. No appreciable binding was observed between the antibodies and physostigmine metabolites or other drugs commonly used with physostigmine. The specificity of the RIA was further validated by measuring physostigmine concentrations in biological samples in tandem with high-pressure liquid chromatography. The RIA was used to study the time course of plasma concentrations and tissue distribution of physostigmine in rats.

Keywords: *Physostigmine; radioimmunoassay; high-pressure liquid chromatography; pharmacokinetics.*

Introduction

Physostigmine, a reversible anticholinesterase, is useful in the treatment of several medical conditions [1–4]. Recently, it has been suggested that physostigmine may be of value in the treatment of cognitive disorders which involve disturbances of memory [5–7]. Physostigmine is also used as one of the preventive measures to block intoxication with irreversible cholinesterase inhibitors [8, 9]. The drug is quite toxic however, and the optimal dose of physostigmine is close to the dose that will produce toxic effects.

Although physostigmine is widely used in clinical medicine, there is no simple and reliable method to measure minute concentrations of the drug in biological fluids. A quantitative assay using human blood has been reported; this assay is based on the duration of cholinesterase inhibition and can detect microgram quantities of physostigmine [10]. A high-pressure liquid chromatography (HPLC) method is available which can detect 50 ng of physostigmine in a biological sample [11]. These methods however are not sufficiently sensitive for the study of the drug's kinetics using plasma and tissue samples. Tritiated physostigmine has been used to study the pharmacokinetics of the

*To whom correspondence should be addressed.

drug in laboratory animals [12]. This method is sensitive but would not be suitable to monitor plasma concentrations of the drug in clinical situations. These observations suggest the need for a sensitive, specific, cost effective method for the monitoring of physostigmine concentrations in biological samples. This paper describes the synthesis of two physostigmine immunogens and the subsequent development of a radioimmunoassay (RIA). Also, a study was undertaken in rats to measure plasma and tissue physostigmine concentrations over time following a single intramuscular dose of the drug.

Experimental

Tritiated physostigmine 19.0 Ci $mmol^{-1}$ was purchased from the Amersham Corp. Physostigmine metabolites were synthesized according to procedures previously reported and were purified by HPLC [13, 14]. Other compounds used in this study were obtained from readily available commercial sources.

Preparation of the immunogens

Physostigmine was conjugated to bovine serum albumin (BSA) by two different procedures (Fig. 1). In the first procedure diazotized *p*-aminobenzoic acid (PABA) was reacted with physostigmine under acidic conditions and the product was coupled to BSA

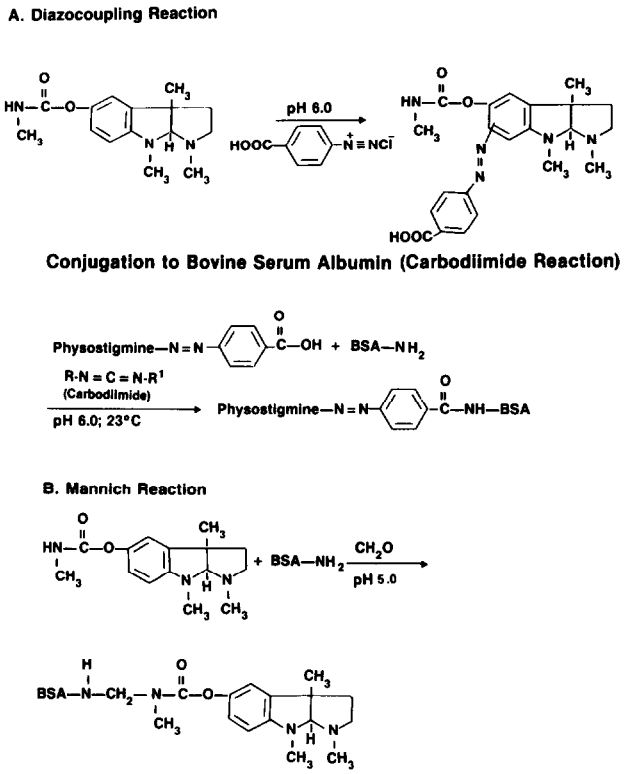

Figure 1
Procedure for the preparation of the physostigmine–BSA immunogens.

with carbodiimide. In the second procedure the Mannich reaction was used to conjugate physostigmine to BSA [15]. The detailed procedures for the preparation of the immunogens are given below.

(a) *Preparation of diazotized physostigmine.* PABA (0.2 mmol) was dissolved in 2 ml of 1 N HCl and the solution was cooled to 4°C in an ice bath. A solution of 0.18 mmol sodium nitrite in 2 ml ice cold distilled water was added dropwise to the PABA solution with constant stirring and while maintaining the solutions at 4°C. The mixture was stirred gently and the reaction was allowed to proceed for 45 min at 4°C. Physostigmine base 0.2 mmol was dissolved in 3 ml 0.1 N HCl and 3 ml of a 50% solution of *N,N*-dimethylformamide; 1.0 μCi of tritiated physostigmine was added. The pH was adjusted to 5.0 and the mixture was cooled to 4°C. The PABA diazonium salt was added dropwise to the physostigmine mixture with constant stirring in an ice bath while maintaining the pH between 5.0–6.0. The reaction mixture was covered to protect the reactants from light and left stirring in the cold room for 4 h.

(b) *Conjugation of the physostigmine–PABA hapten to BSA.* The pH of the physostigmine–PABA hapten was adjusted to 6.0. The hapten was coupled to 40 mg BSA (~0.0006 mmol) in 0.1 mmol sodium phosphate buffer, pH 6.0, in the presence of 0.5 mol 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, stirred overnight at 4°C in the dark and dialysed for 48 h against distilled water with a change of distilled water every 8 h. The average number of the physostigmine (hapten) molecules attached to a BSA molecule was determined to be 12 by radioactive measurement. The assumption was made that the percentage of radioactive physostigmine which was coupled to the BSA was the same as the percentage of non-radioactive physostigmine which was coupled to the BSA.

(c) *Mannich conjugation.* The Mannich conjugation was carried out using procedures previously reported with minor modifications [16]. 100 mg of BSA (~0.0015 mmol) was dissolved in 2 ml of distilled water; 1 ml of 3 M sodium acetate and 3 ml of 8% formaldehyde solution was added. 0.4 mmol of physostigmine base dissolved in 2 ml of 0.3 N HCl and 1.0 μCi of tritiated physostigmine were added to the BSA solution with constant stirring. The pH of the mixture was adjusted to 5.0 and the reaction was allowed to proceed at room temperature for 18 h. The mixture was dialysed against distilled water in a cold room (4°C) for 40 h with a change of water every 8 h. Measurement of radioactivity in the BSA indicated that an average of six molecules of physostigmine were conjugated to each molecule of BSA.

Immunization procedures

Two male New Zealand white rabbits were immunized with each of the immunogens. The immunogens were emulsified with an equal volume of complete Freund's adjuvant. The emulsion containing 0.8 mg of immunogen was injected intracutaneously in multiple dorsal sites. Booster injections were administered every 2 months for the next 6 months using 0.8 mg of immunogen emulsified with incomplete Freund's adjuvant. Antiserum was harvested 1 week after each booster injection.

Radioimmunoassay procedure

The RIAs for standard and unknown samples were always performed in duplicate.

The dilutions of tritiated physostigmine (5000 dpm), antibody (1:150) and standards (100–10,000 pg) were made using the assay buffer (0.01 M sodium phosphate buffer, pH 7.4, and 150 mmol sodium chloride). The total volume of each assay tube was 0.5 ml. After the addition of the antibody, the contents of the tubes were mixed and incubated for 3 h at 4°C. The fraction of physostigmine bound to the antibodies was separated from the unbound physostigmine by the addition of a saturated neutral ammonium sulphate solution [17].

Animal studies

Male Sprague–Dawley rats weighing 500 g were purchased from a commercial laboratory. Physostigmine base was dissolved in a small volume of dilute hydrochloric acid and then diluted with phosphate buffered saline, and the pH of the solution was adjusted to 6.0. A single dose of 0.3 mg kg^{-1} of the physostigmine solution was administered intramuscularly. Blood and tissue samples were taken from the animals at various times after injection: 1, 2, 5, 10, 20, 30, 45, 60, 120, 150 and 180 min. Plasma was separated by centrifugation and stored at −70°C until analysed. Brain, fat, heart, muscle and spleen were removed and frozen on dry ice immediately. On the day of analysis the plasma and tissue samples were thawed. The tissues were weighed and homogenized with an equal volume of a cold solution of 10 mM EDTA, 150 mM NaCl and 0.5% Triton X-100, pH 7.4. The suspension was centrifuged at 15,000 ***g*** in a Sorvall RC-5B centrifuge (Dupont) for 30 min at 4°C. The supernatant was collected and the pellet was washed and centrifuged. The supernatants were combined and filtered through 1 μm pore size filters (RC 60, 9 mm diameter from Schleicher and Schuell Inc., Keene, NH, USA) and 100 μl of the filtrate was injected into the HPLC column.

Specificity of the antibodies

The specificity of the antibodies was tested by the use of HPLC and the RIA in tandem. The HPLC was used to separate physostigmine from its metabolites and related substances, in accordance with procedures previously reported [14]. This method has been used to assay physostigmine in the presence of its metabolites; the satisfactory resolution of the peaks of the metabolites from the physostigmine peak was obtained isocratically. The HPLC system was a Waters Associates equipped with a model 6000A solvent delivery system, a model 441 absorbance detector and a 30 cm μBondapak C-18 column. The column was operated at ambient temperature. After injecting samples into the HPLC column, the effluent was collected every 20 s; the solvent was evaporated using a vacuum centrifuge (Speed Vac Concentrator, Savant Instruments Inc., Hicksville, NY, USA). Each residue was reconstituted using the assay buffer and was analysed for physostigmine by the RIA.

In addition, the specificity of the antibodies was determined by testing the binding of physostigmine metabolites and related compounds to the antibodies. The major metabolites of physostigmine were prepared and purified in accordance with procedures that have been previously reported [13].

Stability of physostigmine in plasma

Physostigmine was added to rat plasma to achieve a final concentration of 36 ng ml^{-1}. The mixture was divided into two parts, one part was maintained at room temperature and the other at 4°C. Aliquots were taken from the mixture for assay of physostigmine at various time intervals.

Results

Both immunogens elicited antibody production in each of the animals injected. The presence of antibody was demonstrated by the binding of tritiated physostigmine to the rabbit serum after the first booster injection. Although the rabbits received several booster injections using each of the immunogens, the titre of antibodies did not change appreciably after the second booster injection. The rabbits injected with the Mannich immunogen produced antibody which had maximum binding in the RIA at a titre of 1:100 dilution. The rabbits injected with the diazo immunogen produced antibodies which were used in the RIA at a titre of 1:150.

Radioimmunoassay

Both immunogens produced antibodies which generated identical standard curves in the RIA. Figure 2 is a typical standard curve obtained by using antibodies from rabbits that were injected with the diazo immunogen. The RIA can detect 100 pg of physostigmine per assay tube using 100 μl of plasma or tissue homogenate (1 ng ml^{-1} of sample). The non-specific binding was always <2% of maximum binding on the standard curve. The physostigmine concentration can be read directly from the standard curve. In each standard curve the percent inhibition of binding produced by 100 pg of physostigmine was significantly different from the binding in the tubes that did not contain physostigmine (paired Student's *t*-test, $P < 0.001$). Neither the addition of plasma nor the supernatant from tissue homogenates had any effect on the standard curve. A 3-h incubation period for the RIA was found to be convenient because the percent of binding did not change appreciably between 3–8 h.

Table 1 demonstrates that more than 95% of the physostigmine can be recovered from plasma samples that are either processed within 15 min at room temperature or kept at 4°C for 3 h. Since the samples were cooled and processed at 4°C, corrections for loss of physostigmine were unnecessary.

The metabolites of physostigmine and related compounds were tested for cross-reactivity. The results are presented in Table 2; the cross-reactivity is expressed as the concentration of a compound necessary to inhibit the binding of the antibodies to tritiated physostigmine by 50% (IC^{50}). It is evident that the affinity of the antibodies for physostigmine metabolites and other related compounds is low (Table 2). Plasma samples and tissue homogenates which had been subjected to HPLC were used in the RIA to verify the specificity of the antibodies. There was only one peak of immuno-reactivity; only those fractions which were eluted at the same time as standard physostigmine were bound to the antibodies (Fig. 3). The slight delay between the peak

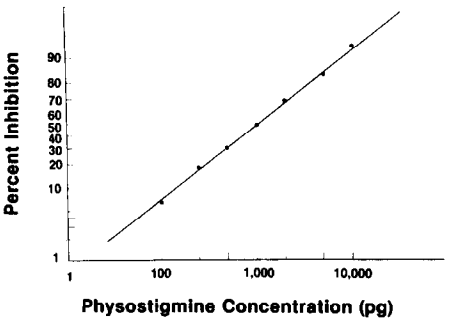

Figure 2
Typical standard curve obtained with both antibodies.

Table 1
Stability of physostigmine in rat plasma

Time	Amount recovered 23°C	4°C
0 min	3.6	3.6
15 min	3.3	3.4
30 min	3.1	3.6
1 h	2.7	3.8
2 h	2.8	3.6
3 h	2.8	3.6
4 h	2.8	3.6

Physostigmine was added to two portions of rat plasma; one-half of the plasma was kept at room temperature and the other was kept at 4°C. Aliquots which originally contained 3.6 ng of physostigmine were assayed at various times.

Table 2
Cross-reactivity of metabolites and related compounds in the physostigmine RIA

Compound	Antibody from diazo-coupled immunogen IC^{50} (ng)	Antibody from Mannich immunogen IC^{50} (ng)
Physostigmine	1.35	1.35
Eseroline	200	200
Rubreserine	224	248
Eserine Brown	550	625
Eserine Blue	600	575
Pyridostigmine	>1000	>1000
Neostigmine	>1000	>1000
Atropine	>1000	>1000
Acetylcholine	>1000	>1000
Melatonin	>1000	>1000
Serotonin	>1000	>1000
Indomethacin	>1000	>1000
Paralidoxime	>1000	>1000

Each compound was tested for cross-reactivity at varying concentrations up to 1000 ng.

of immuno-reactivity and maximum absorbance is due to the time required to pass from the detector to the collection valve.

The accuracy of the RIA was determined by adding 200–4000 pg of physostigmine to a measured volume of pooled normal rat plasma. Aliquots were taken and assayed as unknown samples; each concentration was assayed in quadruplicate. The percentage of measured to added physostigmine ranged from 101 to 106%. The inter-assay and intra-assay coefficient of variation were always <13%.

Use of the RIA to study drug distribution in rats. The concentration of physostigmine in plasma (Fig. 4) and in various tissues (Table 3) was studied at different times following the administration of physostigmine. Physostigmine has a plasma biological half-life of 15 min in rats. The highest tissue concentrations of drug were found in the heart and spleen. The drug levels in the spleen continued to increase for 20 min, while during the same time the drug levels in plasma and other tissues were decreasing.

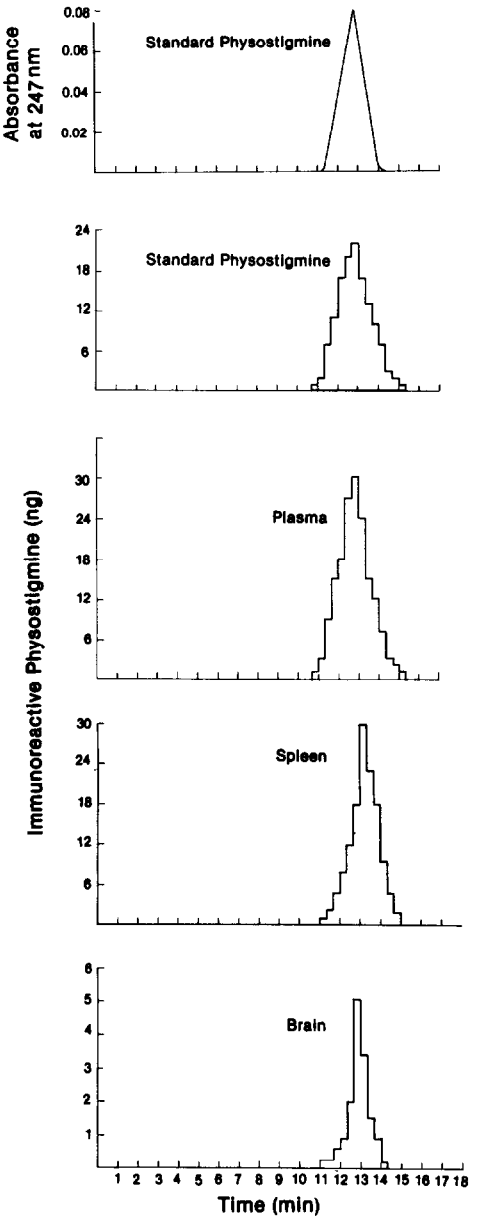

Figure 3
HPLC of standard physostigmine, and tissue homogenates and plasma following intramuscular physostigmine administration. Samples were collected from the column every 20 s for 17 min. The solvent was evaporated and the residue analysed by RIA.

Discussion

RIA procedures are widely used in clinical and research laboratories to quantitate numerous substances in biological fluids. A critical component for the development of a RIA is the production of specific antibodies to a particular compound. This paper describes the production of antibodies to physostigmine using two different immunogens and the development of a sensitive RIA.

The carbamate group is one of the binding groups necessary for both antibodies to bind to physostigmine. When the carbamate group was removed from physostigmine to

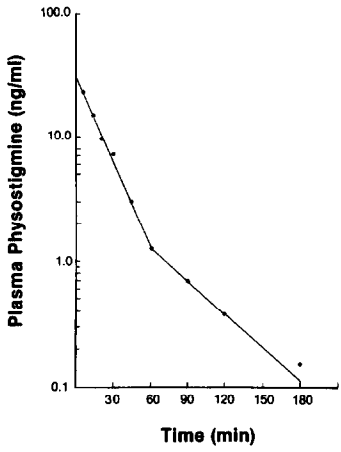

Figure 4
Plasma concentration of physostigmine after intramuscular administration (0.3 mg kg^{-1}). Values are expressed as the mean for three rats.

Table 3
Tissue concentrations of physostigmine (ng g^{-1} of wet wt tissue)

Time interval (min)	Brain	Fat	Heart	Muscle	Spleen
2.5	330.51	230	332.14	320.55	321.84
5	296.61	533.33	785.05	224.74	626.87
10	292.09	397.85	409.45	539.76	955.22
20	197.50	359.74	370.83	322.89	985.71
30	172.94	292.21	212.60	243.37	912.33
45	45.63	95.00	79.23	240.96	834.55
60	15.68	86.31	78.46	86.00	540.00
90	3.05	80.52	66.93	70.00	358.21
120	0.53	58.57	47.24	35.00	226.87
180	0.00	10.34	48.72	37.50	55.56

Tissues were removed, blotted free of blood and homogenized. Aliquots from the homogenate were analysed by the RIA. The tissue concentrations of physostigmine are reported as the mean for three animals at various times after intramuscular administration of 0.3 mg kg^{-1} of physostigmine.

form eseroline almost 150 times as much eseroline was required to displace radioactive physostigmine from the antibodies. The carbamate moiety however is not the only chemical site on the physostigmine molecule which is required for binding to the antibodies because other anticholinesterase inhibitors which contain the carbamate moiety such as neostigmine and pyridostigmine did not bind to the antibodies.

It is important to specifically measure physostigmine because the degradation products are 1000 times less potent than the parent compound in the inhibition of cholinesterase [18] and the binding of small amounts of metabolites to the antibody could result in significant errors in the measurement of the amount of physostigmine-induced anticholinesterase activity present. The HPLC method is specific for physostigmine and none of the metabolites co-elute with the parent drug. The fact that HPLC studies demonstrated that only physostigmine in plasma and tissue homogenate samples bound to the antibody and co-eluted with standard physostigmine is evidence of the specificity of the antibodies. The presence of a single immunoreactive peak in the chromatographs indicates that the antibodies do not bind to any of the metabolites of physostigmine which may have formed in the plasma or tissues.

Using the specific antibodies a sensitive RIA for physostigmine was developed. As little as 1 ng of physostigmine can be detected in 1 ml of plasma or tissue homogenate. The RIA can be used to measure physostigmine levels in dozens of samples during one assay and the results can be available on the same day. Since the antibodies are specific for the parent compound under physiologic conditions, extraction and purification procedures are not necessary. Special equipment or training is not usually required to perform the RIA. Many of these features make the RIA cost effective and suitable for clinical use. The utility of the RIA was validated by following the disposition of physostigmine in rats.

After injection of physostigmine intramuscularly to rats the drug exhibits a biphasic decay curve in plasma. There is a rapid fall in plasma concentration that is followed by a slow decline during the elimination phase. The estimated plasma half-life during the rapid decline phase is approximately 15 min. The volume of distribution is 0.5 l. Physostigmine has a high degree of lipid solubility and is therefore rapidly distributed to central as well as peripheral tissues. Within 2.5 min it is present in all of the tissues studied. The highest tissue concentrations of physostigmine were found in the heart and spleen. The levels of the drug found in brain is consistent with its tertiary amine structure. The high, sustained levels of the drug in the spleen were not expected but may be due to drug binding to cholinesterase in the tissues.

In conclusion, these studies with physostigmine have provided a new, sensitive, and specific RIA procedure which can be used to measure picogram quantities of the drug in biological fluids.

Acknowledgements — The authors wish to thank Mrs Brenda Billingy and Mrs Kim Hargrove for secretarial services.

References

[1] R. C. Duvoisin and R. Katz, *J. Am. Med. Assoc.* **206**, 1963–1965 (1968).
[2] M. B. Walker, *Lancet* **226**, 1200–1201 (1934).
[3] P. Taylor, in *The Pharmacological Basis of Therapeutics,* 7th edn (A. G. Gilman, L. S. Goodman, T. W. Rall and F. Murad, Eds), pp. 110–129. Macmillan Press, New York (1985).
[4] S. M. Aquilonius and U. Hedstrand, *Acta Anaesth. Scand.* **22**, 40–45 (1978).
[5] R. C. Mohs, B. M. Davis, C. A. Johns, A. A. Mathe, B. S. Greenwald, T. B. Horvath and K. L. Davis, *Am. J. Psychiat.* **142**, 28–33 (1985).
[6] K. L. Davis, R. C. Mohs and J. R. Tinklenberg, *Science* **201**, 272–274 (1978).
[7] Y. Stern, M. Sano and R. Mayeux, *Ann. Neurol.* **22**, 306–310 (1987).
[8] J. McGee and H. E. Brezenoff, *Life Sci.* **41**, 65–69 (1987).
[9] W. J. Lennox, L. W. Harris, B. G. Talbot and D. R. Anderson, *Life Sci.* **37**, 793–798 (1985).
[10] S. Ellis, F. L. Plachte and O. H. Straus, *J. Pharmac. Exp. Ther.* **79**, 295–308 (1943).
[11] S. M. Somani and A. Khalique, *J. Analyt. Toxic.* **9**, 71–75 (1985).
[12] S. M. Somani and A. Khalique, *Drug Metab. Dispos.* **15**, 627–633 (1987).
[13] S. Ellis, *J. Pharmac. Exp. Ther.* **79**, 364–372 (1943).
[14] S. T. Yang and L. O. Wilken, *Drug Dev. Indust. Pharm.* **14**, 1061–1078 (1988).
[15] N. S. Ranadive and A. H. Sehon, *Can. J. Biochem.* **45**, 1701–1710 (1967).
[16] A. Levy, K. Kawashima and S. Spector, *Life Sci.* **19**, 1421–1430 (1976).
[17] A. S. Farr, *J. Infect. Dis.* **103**, 239–262 (1958).
[18] B. A. Hensworth and G. B. West, *J. Pharm. Sci.* **59**, 118–120 (1970).

[Received for review 8 February 1989; revised manuscript received 18 April 1989]