J. Pharm. Pharmacol. 1986, 38: 653–658
Received February 12, 1986

Pharmacological evaluation of an injectable prolonged release emulsion of physostigmine in rabbits

SIMON BENITA*, DORON FRIEDMAN AND MARTA WEINSTOCK

Departments of Pharmacy and Pharmacology, School of Pharmacy, The Hebrew University, Faculty of Medicine, P.O. Box 12065, Jerusalem, Israel

Physostigmine was incorporated in an injectable emulsion in an attempt to prolong its pharmacological activity. Emulsions which remained stable over 6 month storage were prepared using optimal experimental conditions. The in-vitro kinetic examination revealed that the rate-determining step in the release process of physostigmine from the emulsion was its partitioning from the oily phase to the external aqueous phase. The in-vivo results indicated that the physostigmine emulsion was able to inhibit the cholinesterase activity for at least 4 h, while the conventional injection inhibited the cholinesterase activity for only 1 to 2 h. The preliminary pharmacokinetic analysis showed that the physostigmine emulsion apparently increased the bioavailability compared with the conventional injectable solution. This could be attributed either to the protection of the sensitive drug from the enzymatic degradation or to improved absorption. The presence of poloxamer micelles in the aqueous phase was shown to enhance the bioavailability of physostigmine without having any effect on its pharmacological activity or duration.

Physostigmine has been shown in both clinical and animal studies to cause a significant reversal of the respiratory depressant effect of morphine without affecting the analgesia (Weinstock et al 1981; Snir-Mor et al 1983). However, its duration of action is short. Physostigmine can also reverse the toxic effects associated with overdosage of other drugs including anticholinergic agents, tricyclic antidepressants (Nattel et al 1979) and benzodiazepines (Larson et al 1977). Again, frequent repeated administration of physostigmine was required because of its short duration of action which has also limited its use in the treatment of Alzheimer's disease (Thal et al 1983). In an attempt to prolong the duration of action of physostigmine, the salicylate was incorporated in a controlled release, injectable emulsion system. The present study was designed to evaluate the release in-vivo of physostigmine from this emulsion.

MATERIALS AND METHODS

Materials

Physostigmine salicylate, soybean oil, crude phospholipids, and mannitol were purchased from Sigma Chemicals Co. (St Louis, Missouri, USA). Polyoxyethylenepolyoxypropylene emulsifier, poloxamer (Pluronic F68) was obtained from BASF (Ludwigshafen, West Germany). The phospholipids were purified according to the method reported by Schubert & Wrethind (1961) before use. This complex emulsifying mixture is composed mainly of phosphatidyl choline and of some anionic phospholipids as determined using standard TLC procedures (Christie 1973).

* Correspondence.

Emulsion preparation and evaluation

The purified phospholipids and physostigmine were dissolved in the oil phase. The non-ionic emulsifier (poloxamer) and mannitol were dissolved in the aqueous phase. Both phases were heated separately to 70 °C and dispersed with a magnetic stirrer. Emulsification was completed using a 'high speed mixer' (Ultraturrax) for 1 min at 85 °C. The resulting fine emulsion was cooled rapidly to below 20 °C. Each emulsion batch was prepared in triplicate.

The effect of the emulsification temperature, the concentration of physostigmine, the surfactant and the nature of the vegetable oil on the stability and the physicochemical properties of the emulsions formed were examined and reported elsewhere (Benita et al 1986).

In-vitro physostigmine release

Determinations of physostigmine release from various solutions and from the emulsions were carried out in an apparatus composed of two compartments

separated by a Nuclepore membrane as shown in Fig. 1. Such a system was derived with modifications from a transdermal in-vitro evaluation system developed by Marty & Wepierre (1982). The emulsion (1 ml) was placed in the donor compartment and the

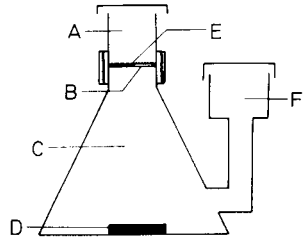

FIG. 1. Experimental system for in-vitro release evaluation of drugs from the emulsions. Key: A, donor compartment; B, Nuclepore membrane filter; C, sink solution; D, stirring bar; E, emulsion delivery system; F, sampling port.

sink solution (50 ml phosphate buffer pH 7·4, prepared according to NF XIV) for releasing the drug, in compartment B. Both compartments were immersed in a constant-temperature water bath at 37 °C. The drug released from the emulsions diffused through the membrane to the sink solution. Samples were taken at different times from the sink solution and the concentration of physostigmine released was determined spectrophotometrically at 245 nm using a calibration curve based on standard solutions in phosphate buffer pH 7·4. The Nuclepore membrane was selected after screening various membranes in diffusion studies using physostigmine salicylate solution, to prevent the membrane from acting as the rate limiting factor in the overall kinetic process.

The various emulsions remained stable over the experimental period of the kinetic process and no oily droplets crossed the membrane.

Pharmacological evaluation

Rabbits were injected intramuscularly (i.m.) with either physostigmine salicylate (0·25 and 0·5 mg kg^{-1}) in 0·90% NaCl (saline) or incorporated into an emulsion (0·5 mg kg^{-1}). Blood was withdrawn from the ear artery before and at 15 min intervals after injection of drugs. The blood was centrifuged immediately for 2 min at 12 000*g* and cholinesterase activity of the plasma was measured by the method of Ellman et al (1961).

An attempt was made to relate the degree of enzyme inhibition quantitatively to the concentration of physostigmine in the plasma of rabbits. Calibration curves were constructed from log concentration of physostigmine and serum enzyme inhibition in-vitro. A linear relation was found over the concentration range tested (Fig. 2). The plasma concentration of physostigmine after intravenous (i.v.) or i.m. injection was then calculated from the degree of enzyme inhibition using these calibration

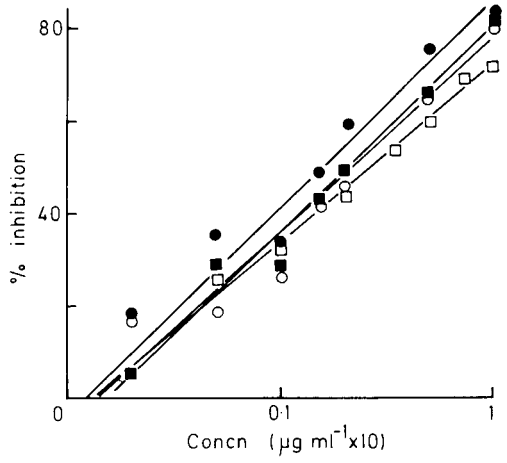

FIG. 2. Effect of plasma physostigmine salicylate concentration on in-vitro cholinesterase activity inhibition (each determination was quadrupled as represented by the four different symbols, to check the reproducibility of the calibration curve).

curves. These data enabled us to perform a preliminary pharmacokinetic analysis of the various solutions and preparations of physostigmine.

RESULTS AND DISCUSSION

The incorporation of physostigmine in the present formulation with the phospholipid as the sole emulsifier caused phase separation. The addition of other drugs to an emulsion for i.v. application (Hansrani et al 1983) also resulted in its reduced stability or cracking (Davis 1974). In a previous study (Benita et al 1986), it was shown that the inclusion of a non-ionic emusifier, poloxamer, was required to stabilize the physostigmine emulsions. The enhanced stabilization was then attributed to the probable formation of a complex interfacial film between the poloxamer and phospholipid molecules at the oil-water interface. This was supported by numerous experimental results presented in the cited study. It should be emphasized that such a combination of emulsifiers has already been used in fat intravenous emulsions and has been found to be free from toxic effects (Meyer et al 1957).

In view of the previous results reported, optimal experimental conditions were found in which the

purified phospholipids formed a resistant close-packed interfacial film with the poloxamer molecules at the oil-water interface of the dispersed droplets and this acted as a mechanical barrier thereby stabilizing the emulsion. Droplet coalescence was also prevented by mutual electrostatic repulsion due to a net negative charge that resided on the droplets. Only minor changes in particle size or zeta potential were observed in these emulsions after six months storage at different temperatures (Benita et al 1986).

In-vitro release of physostigmine

The release of physostigmine from various solutions and emulsions is compared in Fig. 3. The first curve

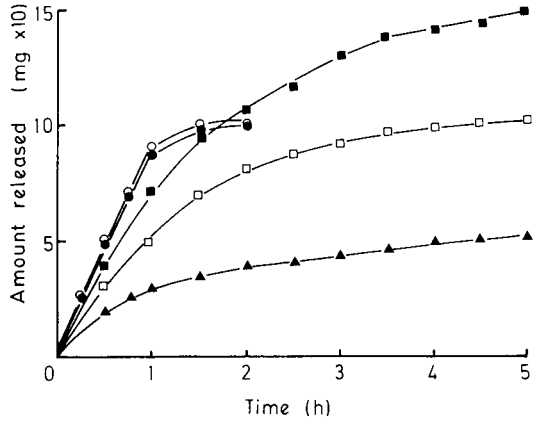

FIG. 3. In-vitro apparent overall release profile of physostigmine from different emulsions and 0·1% solutions (1 ml). Physostigmine concentration refers to the total drug concentration in the emulsion. Key: ○, physostigmine solution; ●, physostigmine solution + poloxamer; ■, emulsion 0·15%; □, emulsion 0·10%; ▲, emulsion 0·05%.

shows the permeation of physostigmine salicylate through the membrane. The process was completed in 1 h.

The critical micelle concentration (CMC) of poloxamer was determined using the technique of surface tension measurements (Attwood & Florence 1983). The CMC value was $2{\cdot}4 \times 10^{-6}$ mol litre^{-1}, close to the value of various other non-ionic emulsifiers reported in the literature.

Different poloxamer concentrations ranging from 5×10^{-4} to 5×10^{-3} mol litre^{-1}, much higher than the CMC, were also studied to find out if the probable incorporation of physostigmine in poloxamer micelles or in the presence of micelles would have any influence on either physostigmine release from micelles or diffusion through the membrane. No influence at all was observed, while the incorporation of physostigmine salicylate into the emulsion decreased its release rate.

These results indicate that the in-vitro release of physostigmine from the emulsion was prolonged compared with that from aqueous solution.

The relatively slow rate of release of physostigmine from the emulsion may be attributed to the retention capacity of the dispersed oily droplets. The release process is dependent on drug partition from the internal oil phase or oil–water interface to the external aqueous phase where it can easily permeate through the Nuclepore membrane to the sink solution (Fig. 3). Eventually, the aqueous phase becomes diluted and as a result, physostigmine transfers from the oily phase to the aqueous phase until a new equilibrium concentration in the latter is reached (i.e. when the physostigmine concentrations are equal in the external emulsion phase and in the sink solution). Thus, the release rate of the drug from the o/w emulsion is mainly a function of the rate of re-establishing equilibrium between both phases and the extent of volume dilution of the aqueous phase in the donor compartment.

The in-vitro release kinetic examination was performed rather as a quality control evaluation than an attempt to correlate in-vitro and in-vivo release of physostigmine from the emulsion.

Pharmacological and preliminary pharmacokinetic evaluation

The in-vivo results shown in Fig. 4 indicated that the physostigmine was released slowly from the emulsion and inhibited the cholinesterase activity for at least 4 h, while the physostigmine conventional injections inhibited the cholinesterase activity for only 1–2 h.

It has previously been reported that a significant antagonism of the respiratory depressant effect of morphine, was only achieved at doses of physostigmine that inhibited plasma cholinesterase by more than 45% (Weinstock et al 1981). Furthermore, under 30% enzyme activity inhibition, no pharmacological action was observed. It must be emphasized that we do not know the extent of inhibition of the enzyme in the various areas of the brain in the rabbit at this level of activity on the plasma enzyme. When the plasma enzyme is inhibited by more than 60%, marked side effects such as fasciculations, salivation, defaecation and tremors are seen. Thus the range of therapeutic efficacy as reported in Fig. 4, without side effects is relatively small, a finding often reported in clinical studies (Thal et al 1983).

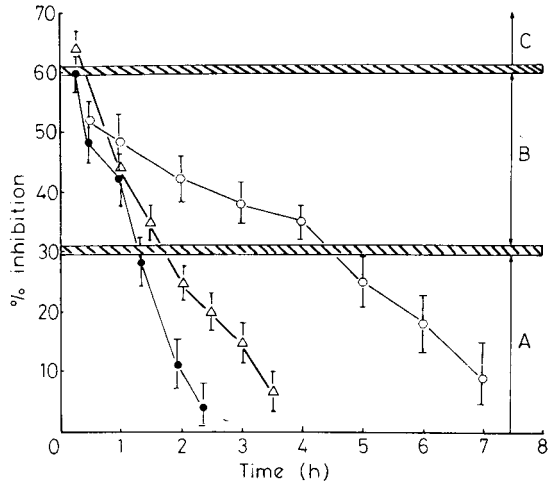

FIG. 4. In-vivo acetylcholinesterase activity inhibition as a function of time of physostigmine salicylate injections (0·25 and 0·5 mg kg^{-1}) compared with physostigmine salicylate emulsion (0·5 mg kg^{-1}) in seven rabbits (formulation: oily phase 20·0, purified phospholipids 1·0, poloxamer 2·0, mannitol 6·0, physostigmine salicylate 0·1 and water to 100·0 g). See text for explanation of the estimated therapeutic range. Key: physostigmine ●, 0·25 mg kg^{-1} i.m.; △, 0·50 mg kg^{-1} i.m. emulsion; ■, 0·50 mg kg^{-1} i.m.; A, ineffective; B, therapeutic; C, toxic.

The concentrations of physostigmine in plasma achieved after injection as extrapolated from the calibration curve of enzyme inhibition as a function of in-vitro concentration are shown in Fig. 5.

It can be seen that the emulsion form of physostigmine yielded prolonged plasma concentrations in

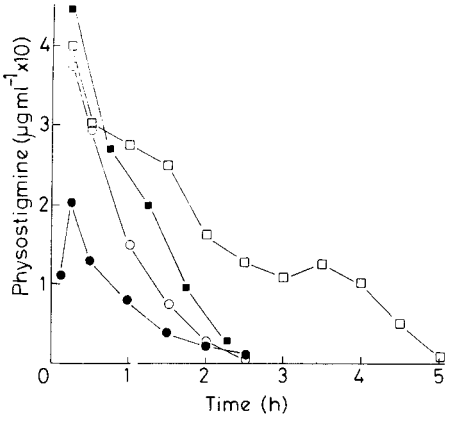

FIG. 5. Plasma physostigmine concentrations following administration of physostigmine solution, 0·10 mg kg^{-1} i.v., 0·25 mg kg^{-1} i.m. and emulsion 0·50 mg kg^{-1} i.m. (each point represents mean of eight rabbits). Key: physostigmine solution ○, 0·10 mg kg^{-1} i.v.; ●, 0·25 mg kg^{-1} i.m.; □, 0·50 mg kg^{-1} i.m. emulsion; ■, 0·50 mg kg^{-1} i.m.

comparison to those achieved after i.m. or i.v. injection of aqueous solution. This is in agreement with previous findings that a subcutaneous injection of an aqueous solution of physostigmine exerted its pharmacological effect for less than 2 h (Koelle 1975).

The dose of physostigmine administered was not the same in the various preparations because of the different in-vivo release profile achieved by each form. For example, 0·5 mg kg^{-1} of physostigmine in the emulsion form produced only mild side-effects whereas the same amount injected in a saline solution i.m. yielded severe toxic reactions. Side effects were minimized by injecting only 0·25 mg kg^{-1} i.m. and 0·1 mg kg^{-1} i.v.

It was not possible to perform a quantitative pharmacokinetic analysis for the evaluation of Ke, first-order elimination constant, or $T_{\frac{1}{2}}$, plasma half-life, of the various physostigmine dosage forms because we did not measure the actual concentration of physostigmine in blood. We could therefore only use the values of the area under the curve (AUC) which were calculated by the trapezoidal method and were considered good approximations, for comparison purposes. AUC, which reflects the overall process or extent of drug absorption, was first calculated for the i.v. dose and then for the other dosage forms. The relative bioavailability of the various physostigmine dosage forms normalized to i.v. administration was then calculated according to the following equation:

$$F = \frac{AUC_T}{AUC_{i.v.}} \times \frac{D_{i.v.}}{D_T} \quad (1)$$

where T refers to the test formulation, i.v. refers to the standard intravenous formulation, AUC is the area under the curve and D is the dose.

The relative bioavailability results are presented in Table 1. The incorporation of physostigmine in the emulsion apparently increased the bioavailability compared with the i.m. conventional injections (Table 1) indicating either that this sensitive drug was afforded more protection from enzyme degradation or spontaneous hydrolysis when given in this form, or that an improved absorption at the injection site took place. The drug is largely destroyed in the body, mainly by hydrolytic cleavage at the ester linkage by cholinesterases; the rate of destruction is related to the concentrations of drug and enzyme.

Physostigmine salicylate in a solution of 2% of the non-ionic poloxamer emulsifier was also injected i.m. to investigate the potential role of the micelles in the in-vivo release or activity of the drug.

Table 1. Apparent bioavailability of intramuscular physostigmine salicylate (phys. sal.) solutions and emulsion[a] in rabbits.

Dosage form	Dose mg kg^{-1}	AUC + s.d. μg ml^{-1} min^{-1} [b]	Bioavailability F
Phys. sal. in saline	0·10 i.v.	19·97 ± 10·32	
Phys. sal. in saline	0·25 i.m.	11·84 ± 2·56	0·24
Phys. sal. in saline	0·50 i.m.	24·20 ± 4·43	0·24
Phys. sal. emulsion	0·50 i.m.	49·12 ± 18·86	0·49
Phys. sal. in 2% poloxamer sol.	0·10 i.m.	10·53 ± 2·4	0·53

[a] Emulsion composition: soybean oil phase 20·0, poloxamer 2·0, phospholipids 1·0, mannitol 6·0, physostigmine salicylate 0·1 and water to 100·0 g.
[b] Mean ± s.d. of 8 rabbits.

The extrapolated plasma concentrations and relative bioavailability are reported in Fig. 6 and Table 1, respectively. It is seen that there was no prolongation of enzyme inhibition by the poloxamer, but a similar kinetic profile to that of physostigmine solution i.m. was observed. In view of the low concentration injected, (0·1%) there must have been a significant increase in bioavailability of physostigmine salicylate in the poloxamer solution.

These results suggested that poloxamer enhanced the absorption of physostigmine injected i.m., but did not affect the pharmacological activity.

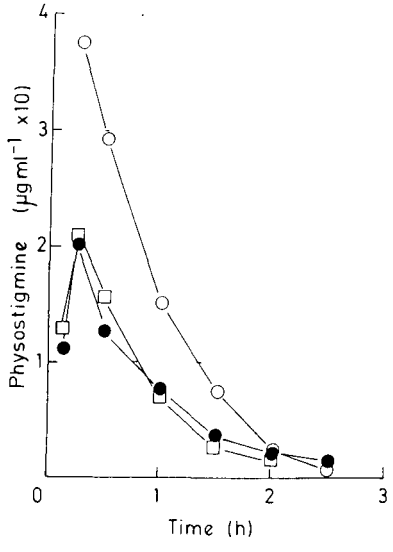

FIG. 6. Effect of route of administration and dosage form on plasma physostigmine concentrations in the rabbit (each point represents the mean of eight rabbits). Key: physostigmine solution ○, 0·10 mg kg^{-1} i.v.; ●, 0·25 mg kg^{-1} i.m.; □, 0·10 mg kg^{-1} i.m. + poloxamer 2%.

It appeared that the prolonged pharmacological activity of physostigmine could be attributed to the incorporation of the drug in the emulsion and not to the presence of the micelles.

Physostigmine dissolved in an oily medium or incorporated at the oil–water interface is expected to be absorbed from the injection site via the external aqueous phase. The transport of physostigmine from the oil phase to the external aqueous phase and its partition coefficient determine the rate of absorption. The influence of these factors on the physostigmine release from the emulsion has already been discussed above. On the other hand, the absorption of physostigmine from the body fluids in the muscle and its appearance in the plasma are rapid and are even enhanced by the presence of poloxamer in the external aqueous phase of the emulsion (Fig. 6).

The prolongation of activity is therefore due to the rate of release of physostigmine from the oily phase. Since the lipid carrier system has been shown to remain for long periods at the injection site (Sesaki et al 1985), the duration of action of physostigmine will therefore depend mainly on the rate of emulsion dilution at the injection site and on its biological degradation. The lipid vehicle of the emulsion slows the release of physostigmine from the injection site. In conclusion, it has been shown that it is possible to prolong the pharmacological activity of a short acting drug by incorporating it in an injectable emulsion delivery system. In view of these findings it may be possible to design a dose regimen of treatment in human subjects with a combination of morphine and physostigmine (in the same delivery system) which would maintain analgesia without concomitant respiratory depression.

The longer acting emulsion form can also be used to antagonize the toxic effect of a variety of other centrally-acting drugs with anticholinergic activity.

Acknowledgements

This work has been supported in part by a grant from the Joint Research Fund of the Hebrew University and Hadassah and in part by a grant from the National Council for Research and Development of Israel.

REFERENCES

Attwood, D., Florence, A. T. (1983) Surfactant systems, Their Chemistry, Pharmacy and Biology, Chapman and Hall, London pp 469–566

Benita, S., Friedman, D., Weinstock, M. (1986) Int. J. Pharm. 30: 47–55

Christie, W. W. (1973) Lipids analysis. Pergamon Press, Oxford

Davis, S. S. (1974) J. Hosp. Pharm. Suppl. 32: 149–160

Ellman, G. L., Courtney, D., Andreas, V., Featherstone, R. H. (1961) Biochem. Pharmacol. 7: 88–95

Hansrani, P. K., Davis, S. S., Groves, M. J. (1983) Parenteral Sci. Tech. 37: 145–150

Koelle, G. B. (1975) in: Goodman, L. S., Gilman, A. (eds) The pharmacological basis of therapeutics, The Macmillan Company New York, 4th Ed. pp 442–465

Larson, G. F., Rutebert, B. J., Wingard, D. W. (1977) Anesth. Analg. 56: 348–351

Marty, J.-P., Wepierre, J. (1982) in: Aiache, J.-M., Devissaguet, J.-Ph., Guyot-Hermann, A. M. (eds) Galénica 2, Biopharmacie, Paris, Techn. and Docu., Lavoissier, pp 454–494

Meyer, C. E., Faucher, J. A., Schurr, P. T., Wester, H. D. (1957) Metabolism 6: 591–596

Nattel, S., Bayne, L., Ruedy, J. (1979) Clin. Pharmacol. Ther. 25: 96–102

Schubert, O., Wrethind, A. (1961) Acta Chir. Scand. Suppl. 278: 1–21

Sesaki, M., Kakutani, T., Hashida, M., Sezaki, H. (1985) J. Pharm. Pharmacol. 37: 461–465

Snir-Mor, I., Weinstock, M., Davidson, J. T., Bahar, M. (1983) Anesthiology 59: 6–9

Thal, L. J., Fuld, P. A., Masur, D. M., Sharpless, N. S. (1983) Ann. Neurol. 13: 491–498

Weinstock, M., Erez, E., Roll, D. (1981) J. Pharmacol. Exp. Ther. 218: 504–508