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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

DIVISION DE NUEVAS CREACIONES

E. S. D.

Re: Expediente No. **07.034.647**

Solicitud de patente a nombre de **PSICOFARMA S.A. DE C.V.**

Yo, DILIA MARIA RODRIGUEZ D'ALEMAN, identificada como aparece al pie de mi firma, domiciliada en la carrera 11 A No. 86-53, piso 6, obrando como apoderada de la sociedad **PSICOFARMA S.A. DE C.V.**, domiciliada en Torielo guerra, C.P. México, solicitante de la patente de la referencia, en respuesta al oficio No. 12843 notificado por fijación en lista el 1 de octubre de 2010 relacionado con el concepto técnico No. 03024, me permito presentar ante ese despacho la siguiente documentación que fue requerida con fundamento en el artículo 46 de la Decisión 486.

- ✓ DINSHEET et al. "Preparation and Evaluation of Muco-adhesive Buccal Tablets of Hydralazine Hydrochloride" Indinan Pharm SCI. Vol 59. no 3, 1997, páginas 135-141.
- ✓ SEGURA-PACHECO et al. "Reactivation of tumor Suppressor Genes by the Cardiovascular Drugs Hydralazine and Procainamide and their potential use" Cancer Therapy, Clinical Cancer Research. Vol. 9, 2003, páginas 1596-1603.
- ✓ BILAC et al. "Studies on Prolonged Release Matrix Tablet Formulations of Hydralazine Hydrochloride" Acta Pharmaceutical Turcica. Vol. 45, páginas 11-15.
- ✓ HIGUCHI T. "Mechanism of Sustained-action Medication. Theoretical analysis of Rate of release of Solid Drugs Dispersed in solid Matrices". J. Pharm SCI. Vol. 52. Diciembre 1963, páginas 1145-1149.

Sobre este particular, nos permitimos manifestar que con la presentación de los anteriores documentos queda total y cabalmente atendido el requerimiento de ese despacho fundamentado en el artículo 46 de la Decisión 486.

Señor Superintendente,


DILIA MARIA RODRIGUEZ D'ALEMAN
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RESEARCH PAPER

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Preparation and Evaluation of Muco-adhesive Buccal Tablets of Hydralazine Hydrochloride

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Multilayered muco-adhesive buccal tablets of hydralazine hydrochloride were developed using polymers, which as Carbopol- 934 and carboxymethylcellulose. Release enhancers such as citric acid, D-mannitol and PEG-4000 and techniques like lyophilization were utilised for optimization of the tablet core. A millipore membrane filter (0.22 μ m) was used satisfactorily to replace animal membrane for drug permeation studies across the membrane.

THE interest in novel routes of drug administration results from their ability to enhance the bioavailability of drugs impaired by a narrow absorption window in the gastrointestinal tract. Optimal therapy requires efficient delivery of therapeutically effective dosage to tissue or organ that needs treatment. The need is not only of reproducibility and predictability of drug release kinetics but also of patient compliance¹. Drug delivery via the buccal mucosa using bioadhesive dosage forms offers such a novel route of drug administration. The route has successfully been tried for the systemic delivery of a number of drug candidates²⁻⁶.

The present study was an attempt to develop a muco-adhesive buccal delivery system for hydralazine hydrochloride (HLZ. HCl), a hypotensive agent. The drug is well absorbed through the gastrointestinal tract but is subjected to extensive first pass metabolism⁷. So, the dose required to produce effective therapeutic serum concentration is relatively high. Since buccal route bypasses first pass metabolism, the dose of HLZ.HCl may be reduced to almost 20% of the oral dose. This in turn would reduce the

dose dependent side effects which are a major limiting factor to its benefits.

The various physico-chemical properties of HLZ.HCl such as low molecular weight (196.64), optimum solubility characteristics, suitable half life (2 to 4 h), dissociation constant (7.1), small dose requirement and absence of objectionable taste and odour make it a suitable candidate for buccal administration^{7,8}.

EXPERIMENTAL

HLZ.HCl was a gift sample from Sarabhai Chemicals Ltd. Carbopol- 934 (CP-934) and hydroxypropylcellulose-M (HPC-M) were obtained as gift samples from Ranbaxy Labs and carboxymethylcellulose (CMC) from Unichem Labs. Citric acid (E-Merck), D-mannitol (BDH) and PEG-4000 (S.D. Fine Chemicals) were obtained from commercial sources. Other solvents and materials used in the study were of reagent grade. Millipore membrane filters of various pore sizes were obtained from Millipore, India.

* For correspondence

Preparation of buccal tablets

The ingredients (HLZ.HCl and polymers) were weighed accurately and mixed by trituration in a glass pestle and mortar. The mix (90 mg) was then compressed using a 9.6 mm diameter die in a hydraulic press (Riken Salki Co. Ltd., Japan) at a pressure of 200 kg/cm² for 30 sec. The tablets were 0.87 to 0.93 mm thick, depending upon the polymer combination used.

For multilayered tablets, the ingredients (100 mg) of the core were compressed using a 9.6 mm die punch set at a pressure of 50 kg/cm² for 10 sec. The core was then removed and placed in the centre of a 13.6 mm die and the ingredients (100 mg) of cap layer poured over it and recompressed at 100 kg/cm² for 15 sec. The upper punch was removed and the mixed ingredients (50 mg) of the backing layer were then added over the cap and compressed at a pressure of 50 kg/cm² for 5 sec.

Lyophilization of polymers

The polymers were taken in a definite ratio (selected by release studies) and dissolved in sufficient quantity of distilled water with stirring. The gel so obtained was pre-frozen and then lyophilized at -70° and stored at -70° for 48 h. The dried solid matrix was then crushed, passed through sieve number 100 and stored in a dessicator till further use.

Water absorption study

This was done on 1% agar gel plates⁹. The tablets were placed with the core facing the gel surface and incubated for 6 h at 37°. The tablets were weighed before and after standing on the agar plate and examined for any physical change.

In vitro dissolution study

Dissolution rate of the drug from buccal tablets was studied using a modified USP dissolution rate test apparatus. The modification consisted of an

internal compartment made up of a 150 ml glass beaker (i.d. 40 mm) into which was placed a teflon cylinder (40 mm diameter, 20 mm height) having a cavity (13 mm diameter, 4mm depth) on one side. The tablet was inserted into the cavity of teflon cylinder so that the core faced the dissolution medium (100 ml isotonic phosphate buffer (IPB) pH 6.6 at 37°). A stirrer was lowered so that it remained at least 1 cm above the tablet surface and stirring was done at 50 rpm. Samples (5 ml) were withdrawn and replaced by fresh dissolution medium every half an hour for 6 h. Filtered samples were then diluted suitably and absorbance was read at the 262 nm. Preliminary studies indicated that the polymers used in the study did not interfere with the estimation of the drug at this wavelength.

In situ diffusion studies

In situ studies of the diffusion of HLZ.HCl from buccal tablets were carried out using the Franz diffusion cell. Fresh bovine cheek pouch membrane was attached to the top of lower compartment. The buccal tablet with core facing the membrane was covered with a glass cap from three sides and lowered in the donor compartment so that the tablet got stuck on the mucous membrane. The receptor compartment was filled with IPB pH 7.4 and constant stirring was done. The assembly was maintained at 37° and stirred magnetically. Samples were withdrawn at 30 min. intervals for 6 h and analyzed as for the dissolution samples.

Millipore membrane filters of 8.0, 1.0, 0.45 and 0.22 µm pore size were also tried for permeation studies instead of mucosal membrane in an attempt to evaluate the suitability of using membrane filters for transmucosal permeation studies. Studies were performed initially on C-4 formulations and finally on optimised formulation also.

In vivo studies of optimized tablets

In vivo studies of the optimized tablets was carried out on healthy, male, white rabbits, weighing

Table 1 : Effect of Polymer Ratio on the Drug Release from Various Muco-adhesive Tablets

Composition	Formula Code														
	A-1	A-2	A-3	A-4	A-5	B-1	B-2	B-3	B-4	B-5	C-1	C-2	C-3	C-4	C-5
HLZ.HCl (mg)	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
CP-934 (mg)	40	26.7	53.3	20	16	40	26.7	53.3	20	16	40	26.7	53.0	20	16
HPMC-K4M (mg)	40	53.7	26.7	60	64	-	-	-	-	-	-	-	-	-	-
PVP-K30 (mg)	-	-	-	-	-	40	53.3	26.7	60	64	-	-	-	-	-
CMC (mg)	-	-	-	-	-	-	-	-	-	-	40	53.3	26.7	60	64
Polymer ratio	1:1	1:2	2:1	1:3	1:4	1:1	1:2	2:1	1:3	1:4	1:1	1:2	2:1	1:3	1:4
Cumulative % drug release* (at 6 hrs.)	24.9	39.3	22.1	43.9	34.1	21.2	38.2	25.6	41.3	49.1	26.3	57.5	37.5	60.4	55.8

* Average of three determinations

one to two kg. The rabbits were fasted overnight and divided into four groups of two rabbits each. Dose of drug was selected on the basis of surface area ratio of rabbits¹⁰. The first group (A) was given an oral dose of HLZ.HCl with the help of plastic tube. Buccal tablet was placed in the cheek pouch of group (B) with the help of spatula. The mount of the rabbits were tied tightly with a cloth to prevent swallowing of dosage form. The blood samples were withdrawn from the marginal ear vein, using xylene for vein dilation. The blood was collected into heparinized tubes and was centrifuged at 1000 rpm for 20 min to separate plasma. To 0.5 ml of freshly prepared plasma was added 1 ml of IPB (pH 7.4) and 6 ml of methanol. This was centrifuged at 850 rpm and to 2 ml of supernatant was added, 1ml of a mixture of buffer and methanol (65:35 v/v). This was filtered through a 0.45 µm filter and 20 µl of this was injected into the liquid chromatographic column using the loop injector. A standard curve of pure drug was prepared by HPLC in the concentration range of 0.1-2.5 µg/ml.

The blood samples were analyzed by reverse phase HPLC with detection by ultraviolet spectros-

copy¹¹. The technique utilised a Waters stainless steel column (30 cm x 3.9 mm) packed with µ Bondapak C. The eluting solution used was 35% of methanol in 0.1 M potassium phosphate buffer (pH 7.4) containing 10 mM EDTA. The solvent flow rate was maintained at 1.5 ml/min. Cumulative drug release was obtained after stipulated time intervals.

RESULTS AND DISCUSSION

The polymers for the preparation of HLZ.HCl buccal tablets were selected on the basis of bioadhesive property, non-toxicity, non-irritancy, stability and compatibility with the drug. CP-934 was combined with either HPMC-K4M, PVP-K30 or CMC. Table 1 gives the cumulative percentage drug release of the buccal tablets prepared using varying ratios of different polymers. Formulation C-4 (containing CP-934 and CMC in the ratio of 1:3) was selected for further study as it had the maximum drug release in 6 h.

Effect of addition of Citric acid

Citric acid was added in a concentration from 2 to 10% in order to enhance the release of drug from

Table 2 : Composition of Optimized Formulation

Composition	Weight in mg of		
	Core	Cap	Backing layer
HLZ.HCl	10.00	—	—
Freezed dried polymer mix of CP-934 :CMC (1:3)	20.00	—	—
CP-934	13.50	66.75	—
CMC	40.50	—	—
Citric acid	6.00	—	—
D-mannitol	8.00	—	—
PEG-4000	2.00	—	—
HPC-M	—	33.25	—
Microcrystalline cellulose	—	—	44.98
Sodium saccharin	—	—	5.00
Tartrazine	—	—	0.02

formulation C-4. The release was enhanced which could be attributed to a decrease in the micro-environment pH of the basic drug (HLZ.HCl) by the addition of citric acid. A concentration of 6% w/w was found to be optimum for maximum drug release.

Effect of addition of D-mannitol

D-mannitol has been used to accelerate the release of drug from polymer matrices¹². It has a sweet taste, a negative heat of solution and dissolution enhancing properties¹³ and hence may be regarded as a suitable excipient for buccal tablets. Maximum release of 78.5% was obtained on addition of 8% D- mannitol to C-4 matrix.

Effect of addition of PEG 4000

Addition of 2% PEG 4000 was found to increase the release of drug to 81.6% from C-4 matrix in 6 h. A further increase in concentration of PEG caused the tablet to erode before 6 h. This is in agreement with earlier studies where PEG has been utilized for development of muco-adhesive erodible buccal

tablets^{14,15}. Hence, 2% PEG-4000 was added to the optimized formulation.

Effect of lyophilization of polymer blend

Use of lyophilized polymer blend of CP-934 and CMC (1:3) upto a concentration of 20% caused an increase in the release of drug from the polymer matrix. However, a further increase in the percentage of lyophilized polymer in the formulation slowed down the release rate.

Development of Optimized formulation

Based on the above studies, a multilayered optimized formulation (Table 2) was developed. The core consisted of the drug, citric acid, D-mannitol, PEG 4000 and a physical mixture alongwith a freeze dried polymer blend of CP-934 and CMC. The cap layer consisted of CP-934 and HPC (2:1) and surrounded the core on three sides so as to allow the release of drug to take place only from the side of core sticking to buccal mucosa. This layer would also aid in the adhesion of tablet to the buccal mucosa

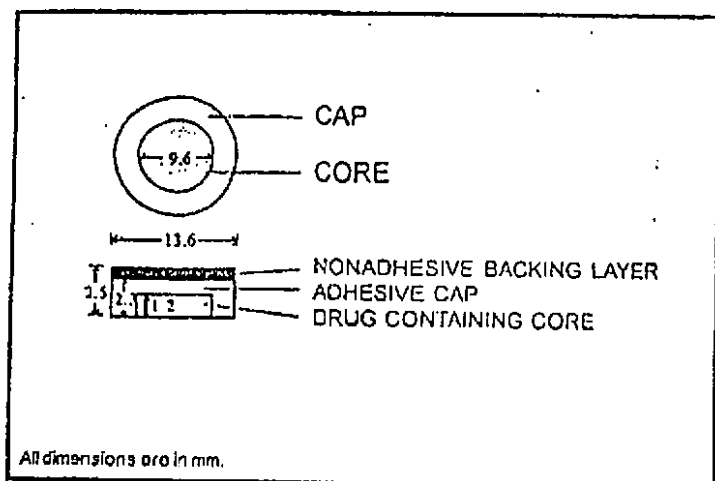


Fig. 1 : Scheme illustration of buccal tablet

for 6 h. The backing layer consisting of microcrystalline cellulose covered the cap layer at the top and overcame the problem of stickiness of the cap layer. It also contained sweetener (sodium saccharin), flavour (lemon oil) and colour (tartrazine) so as to impart an aesthetic appeal to the formulation. Fig. 1 gives a schematic illustration of the buccal tablet.

The tablets were found to be satisfactory when evaluated for weight variation, friability and drug content uniformity. The results of the water absorption study indicated that the tablets did not show any appreciable change in their shape and form during the 6 h, they were kept on agar plate. Swelling of the surface in contact with 1% agar gel was noted and the optimized tablet had a 84.3% gain in weight due to absorbed water after 6 h.

In vitro and *in situ* drug release studies

The optimized formulation exhibited an *in vitro* release of 99.7% (Fig. 2) in 6 h during the dissolution study. When the formulation was subjected to *in situ* diffusion studied using a diffusion cell, a drug permeation of 23.8% was obtained in 6 h across freshly obtained bovine cheek pouch. The correlation between *in vitro* release rate and permeation across

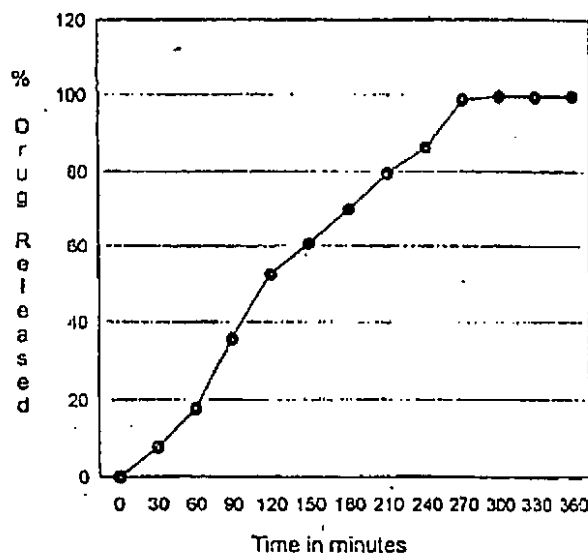


Fig. 2 : *In vitro* dissolution rate study of optimized formulation

the bovine membrane was found to be positive with a correlation coefficient of 0.955.

Analysis of the release rate and permeation rate data showed that the coefficient of variation of zero order rate constant was lower than the corresponding first order values thereby suggesting that the drug release from the optimized formulation may be following zero order pattern.

Comparison of bovine cheek pouch membrane with synthetic membrane

Fig. 3 shows the drug from formulation C-4 permeated through bovine cheek pouch (J-1), millipore filters with pore size 8.0 μm (J-2), 1.0 μm (J-3), 0.45 μm (J-4) and 0.22 μm (J-5). It was seen that permeation obtained from the animal membrane was very close to millipore filter of 0.22 μm . Fig 4 shows the diffusion of drug from the optimized buccal tablet across bovine cheek pouch and 0.22 μm millipore membrane. It appears that the diffusion across the synthetic membrane follows a pattern similar to the animal membrane although the permeation is more (32.5% as compared to 23.8% in 6 h) across the millipore membrane.

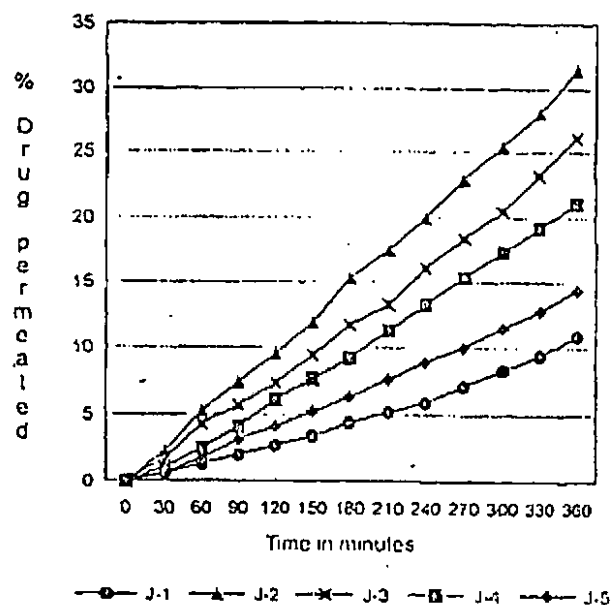


Fig. 3 : Comparison of drug permeation across different types of membranes

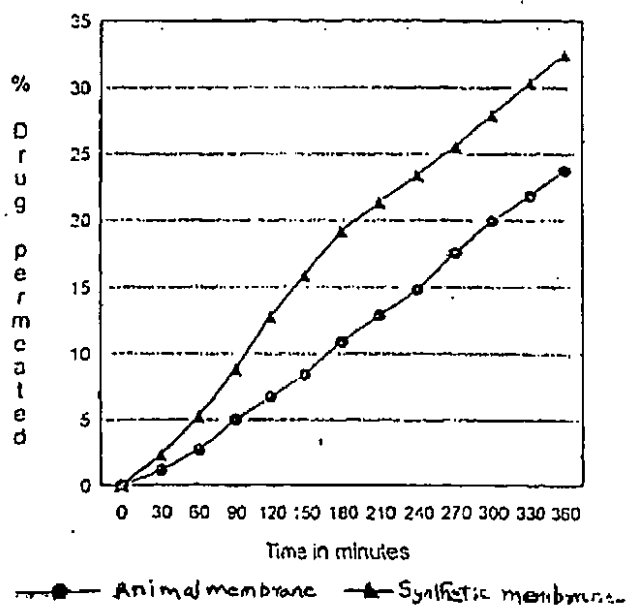


Fig. 4: Comparison of drug permeation across animal and synthetic membrane

In vitro evaluation of the optimized formulation

In vivo evaluation of the optimized formulation in rabbits showed that the drug permeated well across the buccal mucosa. Concentration in blood using equivalent doses of HLZ.HCl via buccal route was found to be the same after 4 h compared to the oral route (Fig. 5).

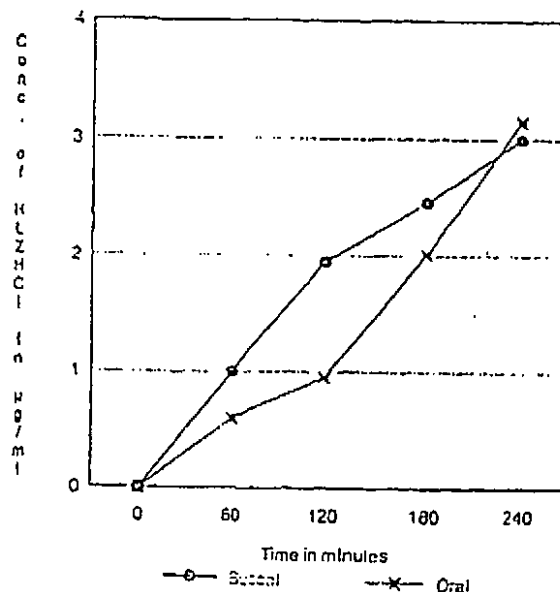


Fig. 5 : *In vitro* release of Hydralazine HCl by oral and buccal route, in rabbits

In conclusion, buccal formulation in the form of multilayered muco-adhesive tablets of HLZ.HCl was developed to a satisfactory level with respect to drug release and bioadhesive strength. Citric acid, D-mannitol and PEG-4000 were found to increase the release of drug from the polymer matrix. A nearly 100% release *in vitro* was obtained using a lyophilized blend of polymers. *In vivo* permeation of drug from buccal tablets in rabbits was also found to be satisfactory.

ACKNOWLEDGEMENTS

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Advances in Brief

Reactivation of Tumor Suppressor Genes by the Cardiovascular Drugs Hydralazine and Procainamide and Their Potential Use in Cancer Therapy¹

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Abstract

Purpose: The purpose of this study is to evaluate the demethylating and tumor suppressor-reactivating activity of hydralazine and procainamide.

Experimental Design: MDA-231, MCF-7, and T24 cell lines were treated for 5 days with 10 μ M hydralazine or 10 μ M procainamide. 5-aza-deoxycytidine at 0.75 μ M was used as positive control. BALB/c nu/nu mice xenografted with MDA-231 cells were treated with these drugs for 7 days by i.p. route. Methylation was assessed by PCR after digestion with methylation-sensitive enzymes for the *ER* gene and with methylation-specific PCR for retinoic acid receptor (*RAR*) β and *p16* genes. Gene expression was evaluated by reverse transcription-PCR and Western blot. The duration of the gene re-expressing effect of hydralazine was analyzed on T24 cells. Functionality of the re-expressed proteins was evaluated by the induction of the estrogen-responsive gene *PS2* on MDA-231 cells and by the induction of *G₁* arrest on T24 cells. The gene demethylating and re-expressing ability of hydralazine was tested in two patients with cervical and head and neck carcinomas, respectively.

Results: Hydralazine and procainamide induced demethylation and re-expression of the *ER*, *RAR* β , and *p16* genes in cultured cells. Both drugs also demethylated and re-expressed the *ER* gene in mice. Hydralazine re-expressed the *p16* gene longer as compared with 5-aza-deoxycytidine. The re-expressed genes were functional. In addition, the

treatment with oral hydralazine demethylated and re-expressed the *RAR* β and *p16* genes in the cervical and head and cancer patients.

Conclusions: These cardiovascular drugs have a promising tumor suppressor-reactivating action and could potentially be used in clinic as an anticancer treatment, most likely to increase the efficacy of current biological or chemotherapeutic treatments.

Introduction

Cancer development is driven from genetic and epigenetic changes at the cellular level that activate and inactivate oncogenes and TSGs,³ respectively. TSGs are inactivated by gene deletions and mutations, however, transcriptional silencing by epigenetic changes is another well-established mechanism of inactivation (1). Thus, methylation at CpG islands suppresses the expression of TSGs such as *p16* (2), retinoblastoma (3), *VHL* (4), *E-cadherin* (5), *hMLH1* (6), *BRCA1* (7), and many others, including the *ER* (2) gene (8). In eukaryotic cells, methylation of cytosines occurs predominantly in genomic regions poor in CpG (9). On the contrary, discrete regions located at the 5' regulatory regions of genes, termed CpG islands, are always unmethylated in normal cells with the exception of transcriptionally silent genes on the inactive X-chromosome (10) and some imprinted genes (11). The knowledge of specific genetic abnormalities in tumors has led to the developing of molecular-targeted therapies as exemplified by gene therapy directed to restoration of TSG activity (12), blocking oncogenes, or their products with either antisense oligonucleotides (13), kinase inhibitors (14), and monoclonal antibodies (15) among other approaches.

Reversion of gene promoter methylation of TSGs is an attractive target for anticancer treatment. A large body of experimental evidence demonstrates that re-expression with demethylating drugs of silenced TSGs leads to a strong inhibitory effects on cancer cell growth *in vitro* and *in vivo* (16). These concepts are supported by the transforming ability of the forced expression of DNA-methyltransferase gene activity (17), as well as by the reversion of the malignant phenotype by antisense oligonucleotides against this gene (18).

These findings pave the way for the clinical testing of demethylating agents in cancer, however, a constrain for their use is the potent carcinogenic and mutagenic properties, as well as the associated toxicity of 5-azacytidine and its analog 5-aza-CdR, two well-known hypomethylating agents (19), being unlikely that these agents will find widespread clinical use. Be-

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³ The abbreviations used are: TSG, tumor suppressor gene; ER, estrogen receptor; 5-aza-CdR, 5-aza-deoxycytidine; RT-PCR, reverse transcription-PCR; *RAR* β , retinoic acid receptor β .

cause of that, there is an urgent need to find out less toxic demethylating agents.

The antihypertensive and antiarrhythmic agents hydralazine and procainamide, respectively, are known inhibitors of DNA methylation that have been used to hypomethylate T cells in experimental systems. This hypomethylation produces overexpression of lymphocyte function-associated antigen 1 that makes T cells autoreactive. This property has been demonstrated *in vitro* and *in vivo* models (20). More recently, Kaplan *et al.* (21) have demonstrated that the lymphocyte function-associated antigen 1 overexpression induced by DNA-methylation inhibitors is caused by demethylation at the *CD11a* gene promoter. Here, we demonstrate that hydralazine and procainamide are able to demethylate and re-express TSGs in experimental and clinical settings.

Materials and Methods

Cell Lines, Tissue Specimens, and Reagents. DMEM culture media and fetal calf serum were purchased from Life Technologies, Inc. (Grand Island, NY). Hydralazine, procainamide, 5-Aza-CdR, and all-*trans*-retinoic acid were obtained from Sigma (St. Louis, MO). MDA-231, MCF-7, and T24 cell lines were obtained from American Type Culture Collection. Cells were grown in DMEM supplemented with 10% FCS at 37°C and 5% CO₂.

5-Aza-CdR, Hydralazine, and Procainamide Treatment. MDA-231, MCF-7, and T24 cells were plated at densities of 5×10^5 cells/100-mm dish, respectively. On day 2, cells were treated with DMEM containing either no drugs or 0.75 μ M 5-Aza-CdR, 10 μ M hydralazine, or 10 μ M procainamide. Drugs were freshly prepared before use. The media containing the drugs was maintained during the 5 days of treatment. On day 6, the cells were harvested using a cell scraper for analysis of gene and protein expression, as well as for DNA methylation assays. These experimental conditions applied for the MDA-231 and T24 cell lines. For MCF-7 cells, the media containing the drugs was changed at day 6, and then, the cells were treated additionally for 24 h with all-*trans*-retinoic acid at a concentration of 1 μ M. For the time course, demethylation experiment MCF-7 and T24 cells were treated with hydralazine for 5 days and harvested for methylation analysis at 24, 48, 72, 96, and 120 h.

RT-PCR. RNA from drug-treated and -untreated cells was obtained using the TriReagent Life Technologies, Inc., RNA extraction kit following the manufacturer instructions. One μ g of total RNA was used for reverse transcription, which was performed with a RT-PCR kit (Perkin-Elmer, Branchburg, NJ) following the manufacturer instructions. For *ER* gene, the PCR reactions were performed as described previously (22). The primers used were 5'-GGAGACATGAGAGCTGCCAAC-3' and 5'-CCAGCAGCATGTCTGAAGATC-3' in a total reaction volume of 20 μ L. The program used was 94°C for 12 min, followed by 24 cycles at 94°C for 1 min, 56°C for 30 s, and 72°C for 40 s. The *p16* gene was amplified as previously described (23) using the primers sense 5'-AGCCTTCGGCTGACTGGCTGG-3' and antisense 5'-CTGCCCATCATCATGACCTGG-3'. For the *RAR β* gene, the conditions were also as previously described (24) using the primers: sense 5'-GACTG-

TATGGATGTTCTGTCAG-3' and antisense 5'-ATTGTGCTCGGCAGACGAAGCA-3'. To control for amount and integrity of the mRNA, the expression of the gene *GAPDH* was also analyzed using primers and conditions already described (24).

Western Blots. For protein analysis, harvested cells were centrifuged, washed with 1× PBS, recentrifuged, and resuspended in radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 7.6), 5 mM EDTA, 150 mM NaCl, and 1% NP40] containing protease inhibitors (10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, and 10 μ g/ml pepstatin A). Fifty μ g of total protein were electrophoresed on a 12% SDS/PAGE gel, transferred to polyvinylidene difluoride membranes, and immunoblotted with the corresponding antibodies: ER; p16; RAR β ; and actin for loading control (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Membranes were incubated with the primary antibody for 1 h at room temperature, followed by incubation with IgG-horseradish peroxidase-conjugated secondary antibodies (Amersham International, Buckinghamshire, United Kingdom) for 30 min at room temperature. Bands were visualized by enhanced chemiluminescence (Amersham International).

Methylation Analysis. The analysis of methylation for the *ER* gene promoter was performed by PCR after digestion of the sample DNA with methylation-sensitive *HpaII* and methylation-insensitive *MspI* enzymes as described previously (25). Briefly, DNA was purified with a Wizard purification kit (Promega, Madison, WI), and then, 1 μ g/sample was digested overnight in a volume of 20 μ L with either 10 units of *MspI* or *HpaII* according to the manufacturer instructions. Then, 2 μ L of the digestion mix, equivalent to 100 ng of DNA, were then amplified in a volume of 20 μ L using the primer sequences 5'-TCTCCCCTCACTCCCCTGC-3' and 5'-GAAATCAAAACAAGCCTACCC-3' that amplify a product of 292 bp of the p0 *ER* gene promoter. The protocol of amplification consisted of 95°C for 5 min, 25 cycles of 94°C for 1 min, annealing at 60°C for 1 min, and extension at 70°C for 1 min, followed by a final extension of 5 min at 72°C.

Absence of bands from samples digested with *MspI* indicate that the digestion worked as this enzyme cuts irrespective of the methylation status, no amplification in reactions digested with *HpaII* is indicative of demethylation, whereas amplification indicates methylation.

Methylation status of *p16* and *RAR β* genes was analyzed by methylation-specific PCR as described previously (26, 27). Briefly, 1 μ g of DNA in a volume of 100 μ L of each sample was denatured with freshly prepared NaOH at a final concentration of 0.2 M and modified according to the manufacturer instructions of the DNA Modification kit (Intergen, Purchase, NY). The PCR mixture contained 2 μ L of 10× PCR buffer, 0.5 units of Taq Gold polymerase, deoxynucleotide triphosphates (each 1.25 mM), 300 ng of primers/reaction, and bisulfite-modified DNA or unmodified DNA in a final volume of 20 μ L. Products were visualized in a 2% agarose gel under UV light.

In Vivo Experiments. To evaluate whether hydralazine and procainamide demethylate re-express the *ER* gene *in vivo*, 16 nu/nu 8-week-old female mice (Harlan Teklad, Madison, WI) were injected with 6×10^6 MDA-231 cells in the flank. After 4–8 weeks, once the tumors reached a size between 0.5 and 1 cm in diameter, 4 mice/group were injected by i.p. route

with 200 μ l of normal saline, 300 μ g of 5-Aza-CdR, 5 mg/kg hydralazine, or 20 mg/kg procainamide daily for 7 days. At day 8, mice were sacrificed under chloroform anesthesia and tumors removed. Tumors were immediately processed for protein, RNA and DNA extraction, and then for evaluation of the *ER* gene and product expression, as well as the assessment of the methylation status as described above.

Heritability of DNA Demethylation. To evaluate the duration of the gene-reactivating effect of hydralazine as compared with 5-Aza-CdR, T24 cells were treated with either of these drugs for 5 days as described above. At day 6, the media was changed, and cells were successively passaged in drug-free medium. Every subsequent day cells were analyzed for the mRNA of the *p16* gene by RT-PCR.

Functional Assays of the Re-expressed Products of *ER* and *p16* Genes. To evaluate whether the re-expressed genes were functional, the MDA-231 and T24 cell lines were treated in culture as above described with hydralazine or 5-Aza-CdR. For the breast cancer cell line, the media containing the drugs was replaced by fresh media without phenol red (phenol red-free RPMI) containing 10% dextran charcoal-stripped FCS. After 24 h in these latter conditions, estradiol at a concentration of 10 nM was added for additional 24 h, and then, cells were analyzed for the expression of the estrogen-responsive gene *PS2* as described previously (22). For the bladder cancer cell line, cells were kept for 24 h in drug-free medium after treatment (confluency ~80%) and then harvested and fixed in 75% ethanol, stained with propidium iodide, and analyzed by flow cytometry to determine the cell cycle profile.

Clinical Evaluation of the Demethylating and Gene Re-expression Activities of Hydralazine. To evaluate whether hydralazine was able to demethylate and reactivate expression of silenced TSGs in a clinical setting, two patients (one with cervical cancer and another with head and neck cancer) who had progressed to all available treatment were treated with hydralazine after a signed informed consent. Hydralazine was administered for 10 days by oral route (50 mg 3 times a day). Two days before commencing treatment, a punch biopsy from the tumors was taken for DNA and RNA extraction to document methylation and lack of expression of the *RAR β* and *p16* genes, respectively. At day 11, a biopsy from the same site was taken for reanalysis of methylation and gene expression. To confirm the changes in methylation pre- and posttreatment, the PCR products of the *RAR β* gene were sequenced in an ABI PRISM 310 (PE Biosystems) sequencer.

Results

Previous studies have revealed that the *ER*, *p16*, and *RAR β* genes are methylated in MDA-231, T24, and MCF-7 cell lines, respectively, and also that 5-Aza-CdR is able to demethylate these genes. To determine whether other inhibitors of DNA-methyltransferase can demethylate TSGs, these cell lines were treated with hydralazine and procainamide at indicated doses for 5 days. As a positive control, 5-Aza-CdR was used. As expected, the PCR from untreated MDA-231 cells was negative in the sample digested with *Msp*I but positive in the sample digested with *Hpa*II, indicating cells were methylated. On the contrary, the PCR failed to amplify after digestion with *Hpa*II

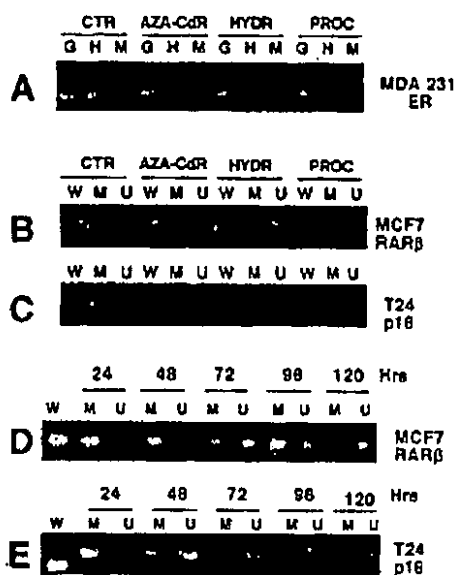


Fig. 1 Methylation analysis of the *ER*, *RAR β* , and *p16* genes. A, G is undigested genomic DNA as positive control, H is *Hpa*II, and M is *Msp*I. The band in Lane 2 indicates lack of digestion with *Hpa*II and, therefore, methylation of untreated MDA-231 cells. B and C show the analysis by methylation-specific PCR of *RAR β* and *p16* genes in MCF-7 and T24 cells. W is wild type, M is methylated primers, and U is unmethylated primers. D and E show a time course experiment of *RAR β* and *p16* gene demethylation over 5 days on the MCF-7 and T24 cells, respectively.

from cells treated with the three demethylating drugs, which indicates demethylation (Fig. 1A). To investigate whether the demethylating ability of hydralazine and procainamide was not cell line nor gene specific, T24 and MCF-7 cells were treated with these demethylating drugs. The methylation-specific PCR using bisulfite-modified DNA for the *p16* and *RAR β* genes demonstrated methylation in untreated but demethylation in treated cells (Fig. 1, B and C). In addition, the time course demethylation analysis done in MCF-7 and T24 cells for the *RAR β* and *p16* genes with hydralazine showed that as expected, the unmethylated band appeared after 48 h (Fig. 1, D and E). Interestingly, the demethylation was faster for the *p16* gene. Thus far, these results demonstrated that hydralazine and procainamide were able to demethylate these TSGs. To investigate whether demethylation correlated with gene reactivation, the expression of these genes was investigated at the level of RNA and protein. Untreated cell lines were negative, but after 5 days of treatment with the drugs, the cells expressed the transcript and protein (Fig. 2, A–C). To further explore the ability of hydralazine and procainamide to induce demethylation *in vivo*, nu/nu mice received injections of MDA-231 cells to generate tumors and, subsequently, were treated for 7 days by i.p. injections of hydralazine or procainamide. The analysis of the expression of the *ER* gene at protein and RNA levels as well as the methylation status of the gene demonstrated that both drugs were able to demethylate and re-express the *ER* gene (Fig. 3). Thus far, these results demonstrated that like 5-Aza-CdR, both

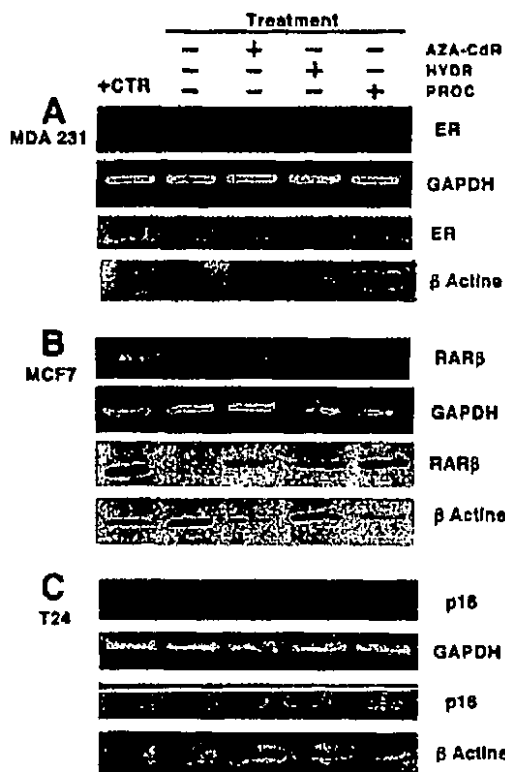


Fig. 2 Expression of the mRNA and proteins of A, ER (MDA-231); B, RARβ (MCF-7); and C, p16 (T24) genes by RT-PCR and Western blot. The positive control (+ctr) for A is MCF-7, for B is MDA-231 exposed to 5-aza-CdR/At-RA, and for C is HeLa cells.

drugs demethylate and re-express TSGs *in vitro* and *in vivo*. To compare the effect on the heritability of these changes, hydralazine and 5-Aza-CdR were tested in the T24 cell line. The results showed that under identical conditions of treatment at the doses tested, hydralazine kept longer the reactivating effect on the *p16* gene (Fig. 4).

It has been demonstrated that genes reactivated after 5-Aza-CdR treatment are functional. Because of the importance of this phenomenon upon the potential therapeutic use of demethylating agents, we tested whether the reactivated genes by hydralazine were functional. As it could be predicted, in the MDA-231 cell line, the re-expressed ER product induced the transcription of the *PS2* gene upon stimulation for 24 h with estradiol (Fig. 5A). Likewise, in the bladder cancer cell line, the reactivation of *p16* gene was able to induce G₁ arrest, indicating that the gene product was functional (Fig. 5B).

It has been reported that 5-azacytidine selectively increases δ-globin synthesis in a patient with β Thalassemia (28), therefore, we treated two cancer patients with hydralazine for 10 days to confirm if our experimental results were clinically achievable. Fig. 6 shows that hydralazine demethylated and re-expressed the *p16* gene in the head and neck cancer patient (Fig. 6A) and the *RARβ* gene in the cervical cancer patient (Fig. 6B). This demethylation was confirmed by sequencing the *RARβ* gene prod-

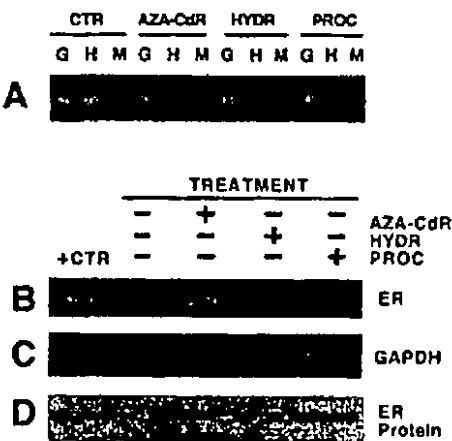


Fig. 3 Analysis of methylation and product expression of the ER gene in nude mice. A shows that the tumor of control mice were methylated as shown by the band in the Lane 2 indicating no digestion with *HpaII*. The band disappeared with the treatment with 5-aza-CdR and hydralazine, which indicates gene demethylation. Lane 11 corresponds to the mice treated with procainamide. The band is absent in *MspI* but is very weak in *HpaII*, indicating that was only partially demethylated; B and C are the product expression by RT-PCR and Western blot. In both cases, the intensity of bands in procainamide is weaker, correlating with a partial demethylation. G is undigested genomic DNA.

ucts (methylated pretreatment and demethylated posttreatment). The product amplified encompasses position 950-1095 of the promoter-exon region of this gene (GenBank accession no. X56849 as shown in Ref. 27), which has 10 methylated cytosines. All these were demethylated after treatment with hydralazine. Fig. 6, C and D, shows position 1000-1095 in which the five methylated cytosines marked with the arrow became demethylated.

Discussion

The demonstration that the re-expression of TSGs silenced by methylation can have therapeutic value in malignant diseases has been hampered by the unavailability of suitable drugs for clinical testing. In this study, we show that two noncytotoxic agents having current clinical use for nonmalignant conditions are able not only to demethylate but to re-express *in vitro* and *in vivo* the functional products of TSGs in cancer cell lines. In addition, we demonstrate that this effect is clinically achievable by hydralazine.

In this work, we used the ER-negative MDA-231 cell line that lacks ER gene expression because of hypermethylation of its promoter (29, 30). It has been shown that the demethylating agent 5-aza-CdR is able to re-express a functional ER protein (8). Our results demonstrate that untreated MDA-231 cells have methylated and silenced the expression of the ER gene, however, after 5 days of exposure to procainamide or hydralazine, this epigenetic change was reverted. Methylation-specific PCR after bisulfite modification is currently considered as the most simple and effective method to determine the methylation status of gene promoters. The ER gene methylation has been analyzed with this technique (31), however, we were unsuccessful in

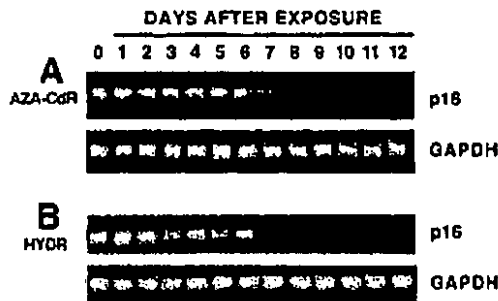


Fig. 4 Expression of p16mRNA in T24 cells treated with 5-Aza-CdR (A) and hydralazine (B). Day 0 is the fifth day of drug treatment. A faint band is still seen at day 11 with hydralazine as compared with day 8 with 5-Aza-CdR.

reproducing these results, therefore, we decided to analyze the *ER* gene methylation by PCR amplification after digestion with methylation-sensitive enzymes. This technique has some technical limitations that mostly depend on an efficient enzymatic digestion, however, our data clearly demonstrate that this was not the case because no bands could be amplified after digestion with *Hpa*II from treated cells, but they were present in untreated cells. In addition, these results are supported by the identical results obtained with 5-aza-CdR, used as a positive control.

To rule out that the effect of procainamide and hydralazine could be a cell line or a gene-specific effect, we studied the *p16* gene in the bladder cancer cell line T24. This cell cycle regulatory gene has been widely studied in regard to its methylation status in cancer. Previous results in this cell line have shown that 5-aza-CdR reverts its methylation and induces its re-expression *in vitro* and *in vivo* (16). Our results indicate that procainamide and hydralazine are also able to re-express its gene product and also provide evidence that the reactivation of the *p16* gene is because of demethylation as demonstrated by methylation-specific PCR. In addition, our results demonstrate that the gene products expressed were functional as shown by the induction of the estrogen-responsive *PS2* gene after estradiol treatment and to the increase in the population of T24 cells in the *G*₁ phase of the cell cycle. These findings additionally support the potential use of hydralazine for reactivate TSGs in cancer.

To provide further evidence, we also studied in the MCF-7 cell line the effects of these two drugs on the *RARβ* gene. This gene has been found silenced by methylation in several cell lines, as well as in primary tumors such as lung carcinoma where the methylation of this gene has prognostic implications (32). It is known that the expression of the *RARβ* gene is up-regulated by retinoic acid (33) and that *in vitro*, demethylation of the *RARβ* gene is not sufficient for its expression unless cells are treated with retinoic acid (34). Our results show that after 5-day exposure to the tested drugs, all-*trans*-retinoic acid induced the expression of the receptor in the treated cells and that this effect correlated with promoter demethylation.

An important finding of this study was that the systemic administration of hydralazine and procainamide to animals was able to demethylate and re-express the *ER* gene without over toxicity. Interestingly, the concentration of these two drugs at

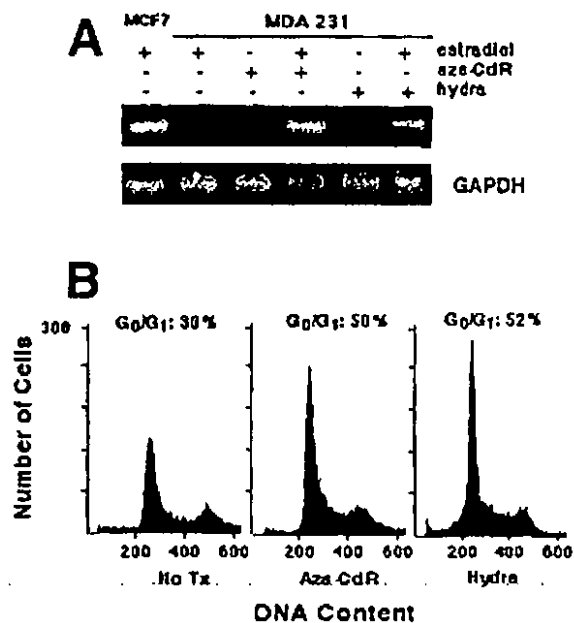
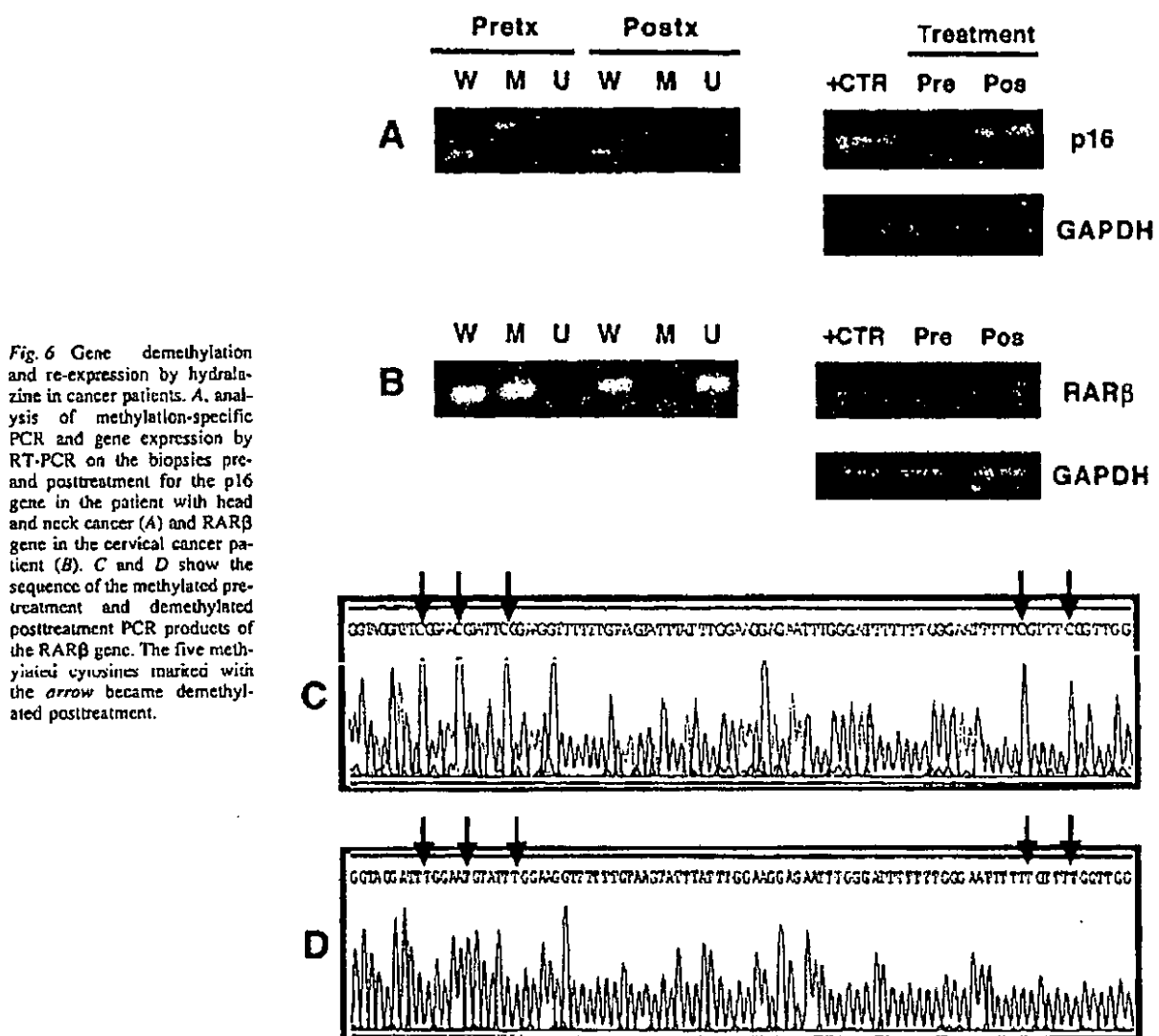


Fig. 5 Functionality of expressed genes. A shows that estradiol induced *PS2* gene on cells previously exposed to either 5-aza-CdR or hydralazine, and B shows cell cycle profile of T24 cells exposed to the drugs. 5-aza-CdR and hydralazine were equally effective on inducing *G*₀/*G*₁ arrest.

which the effect was observed *in vitro* and *in vivo* is in the therapeutic range used in clinic as antiarrhythmic and antihypertensive, respectively (35, 36).

It is known that 5-aza-CdR inhibits cytosine methylation by covalently binding DNA methyltransferase and that its demethylating effect is maintained for at least nine doubling times, being after this time, a gradual recuperation of methylation to the original level (37). The mechanism by which hydralazine and procainamide inhibit DNA methylation is still unknown, however, both drugs bind deoxyguanine-deoxycytosine rich DNA sequences (38), and this binding may interfere with translocation of the enzyme DNA-methyltransferase along the DNA strand. Accordingly, the demethylating effect of both drugs is comparable with 5-aza-CdR when evaluated by the methyl-methionine assay and by direct quantification of deoxymethylcytosine (39).

Because procainamide and hydralazine most likely have a distinct mechanism of demethylation than 5-aza-CdR, we analyzed the effect on inheritability of the re-expression upon the *p16* gene in the T24 cell line. Surprisingly, hydralazine was more effective than 5-aza-CdR for keeping re-expressed this gene longer, which is in line with a delayed demethylating effect of this drug when tested *in vitro* (39). The evidence would suggest that to keep genes demethylated, cells would need to be in continuous exposure to the demethylating drugs, which from a practical point of view would favor the use of hydralazine instead of 5-aza-CdR because this antihypertensive drug is given in daily basis for long periods of time. An important issue to be addressed is whether the sole re-expression of the sup-



The implication of this study seems important because these drugs could have a role in cancer therapy. It is remarkable their known negligible toxicity as well as their availability for oral use and low cost. Currently, there are at least two clinical Phase I-II trials ongoing, testing the demethylating ability of 5-aza-CdR in patients with advanced malignancies, as well as

preclinical testing of antisense molecules targeting the DNA-methyltransferase gene. The characteristics above describing hydralazine and procainamide make them suitable for clinical trials and seem a simple alternative to 5-aza-CdR and antisense oligonucleotides.

Despite that both hydralazine and procainamide were able to demethylate and reactivate the TSGs tested, we chose to perform additional experiments only in the former as our results in mice suggested that procainamide achieved only partial demethylation, however, the potency of their demethylating activity should be formally compared. In addition, because the clinical use of an antiarrhythmic agent could be more problematic than of an antihypertensive in noncardiovascular conditions, hydralazine seems to be more appropriate for additional testing.

In conclusion, we provide evidence that these two cardiovascular drugs reactivate TSGs silenced by methylation and may become a potential useful therapy targeting one of the most common epigenetic abnormality found in cancer cells. Just to mention some of their potential applications, in the case of breast cancer, for instance, the re-expression of the ER protein could make patients with ER-negative tumors responsive to endocrine management, and in the same way, the efficacy of retinoid acid treatment of some tumor types could be increased by re-expressing the RAR β through its demethylation.

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Studies on Prolonged Release Matrix Tablet Formulations of Hydralazine Hydrochloride

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Abstract

The purpose of this study was to prepare prolonged release tablet formulations of hydralazine hydrochloride which has been used as an antihypertensive agent. To achieve this, carbomers (Carbopol® 834, 934, 940 and 1342) were used as polymer in different concentrations. Magnesium stearate was used as lubricant and direct compression method was applied for preparing tablets.

Tests on tablets prepared according to different formulations were carried out in respect of their content uniformity, diameter-height ratio, weight deviation, hardness, friability and *in vitro* dissolution rate of the drug. Spectrophotometric method was used for hydralazine hydrochloride assay. USP XXII basket method was performed for the *in vitro* dissolution studies and dissolution data were evaluated kinetically.

Key words : Hydralazine hydrochloride, prolonged release, matrix tablet, Carbopol®

Introduction

Hydralazine hydrochloride (Hyd.HCl) effects on veins directly as a dilatator is as an antihypertensive agent. By reducing periferic vein resistance, relaxing the plain muscles of arteriols and increasing the renal blood flow, it is preferred to the other hypertension agents. In addition, it reduces the resistance of veins in the kidneys thus providing blood flow at sufficient level during medical treatments.

Hyd.HCl is metabolized very rapidly with a half-life about 2-4 hours. Treatment starts with a dose of 40 mg which is given by 3-4 times in a day. Unless is used with a beta blocker such as propranolol, it causes tachicardy and palpitation. If the patient uses propranolol besides Hyd.HCl, the dose can be increased to 100 mg and also according to the response the dose can be increased up to 200-400 mg daily (Martindale XXX, USP 1990, Rawlins 1977, Kayaalp 1988). Spectrophotometric and titrimetric methods have been used for the assay of Hyd.HCl (Chester *et.al.* 1979, Clarke E.G.C. 1981).

In recent years the use of polymers for the purpose of controlling the drug release has become an important subject. A wide range of polymers can be employed among which the group of acrylic acid derivatives, known officially as carbomers or by their commercial name Carbopol® were included (Perez-Marcos *et al.* 1991, Malley *et. al.* 1987) prepared controlled release matrix tablet of sodium salicylate using Carbopol® 934. Their studies carried out with thin sheets of various concentrations of sodium salicylate pressed into Carbopol® matrix. (Şenel *et. al.*, 1991) studied the factors effecting the formulation of sustained release potassium chloride tablets. Polyvinylchloride and methylcellulose were used as matrix materials.

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The in vitro release of lithium carbonate incorporated into polymethylmetacrylate, polyvinylchloride, hydrogenated vegetable oil and carbomer was studied by (Çiftçi *et al.*, 1990). The formulation containing 10%(w/w) carbomer showed sustained release profile comparable to that of the standard. Hydrogenated vegetable oil and Carbopol® have been used separately in the production of sustained release systems as insoluble and erodible matrices for oral administration. The release rate changes are explained on the bases of interaction between the gel and other matrix components in the presence of water (Malamataris and Ganderlon 1991).

In this study, prolonged release matrix tablet formulations of Hyd.HCl elaborated using the direct compression technique were prepared. To achieve this, Carbopols® 834, 934, 940, 1342, were used as polymer in different concentrations (40-50%).

Materials and Methods

Hydralazine hydrochloride Ciba-Geigy Istanbul, Turkey; Carbopol® 834, 934, 940, 1342 Goodrich, USA; Magnesium stearate; E.Merck Germany.

Preparation of the tablets: Formulations were prepared by direct compression technique Magnesium stearate (5%) and Carbopol® (834, 934, 940 and 1342) in different concentrations (40-50%) were used as lubricant and polymer respectively. Tablets weighing 105 mg were compressed using by single punch tablet machine, according to the classical tableting procedure. The formulation codes and contents are shown in Table 1.

Hydralazine hydrochloride content: A spectrophotometric method was used for the Hyd.HCl assay. 25 mg of Hyd.HCl was accurately weighed and dissolved in water and the volume was adjusted to 100 ml. Ten samples of 1-5.5 ml (0.5 ml intervals) were taken from this stock solution and diluted to 50 ml with water. Absorbances were measured spectrophotometrically at 305 nm against water. Regression equation and regression coefficients were calculated.

Table 1. Matrix tablet formulations of hydralazine hydrochloride

Contents	Tablet code							
	F1	F2	F3	F4	F5	F6	F7	F8
Hydralazine hydrochloride (mg)	69.3	69.3	69.3	69.3	69.3	69.3	69.3	69.3
Carbopol® 834 (w/w %)	50	-	-	-	40	-	-	-
Carbopol® 1342 (w/w %)	-	50	-	-	-	40	-	-
Carbopol® 940 (w/w	-	-	50	-	-	-	40	-
Carbopol® 934 (w/w	-	-	-	50	-	-	-	40
Magnesium stearate(w/w %)	5	5	5	5	5	5	5	5

Crushing strength: Ten tablets were tested using Monsanto hardness apparatus.

Friability: Twenty tablets were weighed and rotated for 100 revolutions for 4 min in a Roche friabilator (TS 1). The tablets were then reweighed and the percentage of friability calculated.

Diameter-height ratio: This ratio was determined using a micrometer.

In vitro dissolution test: Dissolution tests were performed according to the basket method described in USP XXII (100rpm, 37 C° ± 0.5). Dissolution studies were carried out in 900 ml of simulated gastric (SGM) and intestinal medium (SIM) without enzymes for a period of 8 hours. The absorbances of the samples were measured spectrophotometrically at 305 nm against blank. The amounts of Hyd.HCl in the samples were calculated using the standard curve equation.

The dissolution data were evaluated kinetically according to zero order, first-order, Hixon-Crowell, RRSBW, Q square root of time and Higuchi equations. The release rate constants (k), and determination coefficients (r^2) were calculated by means of a computer program (Ağabeyoğlu 1984).

Results and Discussion

Content uniformity, friability, hardness, and diameter-height ratio results of tablets were in accordance with the pharmacopoeial limits (USP XXII). The results are presented in Table 2.

Table 2. Content uniformity, friability, hardness, and diameter-height ratio results of tablets (n= 10)

Parameters	Tablet code							
	F 1	F 2	F 3	F 4	F 5	F 6	F 7	F 8
Hydralazine hydrochloride(mg)	68.6	70.1	71.3	71.9	70.4	70.9	71.1	70.1
Tablet weight (mg)	104.08	105.02	105.42	105.42	104.52	98.87	98.69	98.68
Weight deviation (%)	± 2.32	± 1.44	± 1.06	± 1.90	± 0.58	±0.76	± 0.87	±0.73
Hardness (kg)	4.50	4.20	2.50	2.00	1.50	4.75	2.50	2.00
Friability (%)	0.08	0.29	0.08	0.21	0.22	0.16	0.06	0.26
Diameter-height ratio	5.06	5.06	4.90	4.76	5.06	4.90	5.06	4.90

Table 3. Dissolution results of formulations

Time (min)	Released %							
	F 1	F 2	F 3	F 4	F 5	F 6	F 7	F 8
30	27.48	24.14	27.11	30.20	28.04	24.40	26.62	28.64
60	42.23	36.40	49.64	44.68	45.09	41.71	58.08	44.71
120	65.72	65.18	97.22	69.85	69.85	78.11	99.15	83.85
180	82.91	78.71	-	86.99	95.42	82.66	-	88.64
240	88.94	84.44	-	96.03	-	86.67	-	95.67
360	94.05	90.25	-	97.48	-	90.02	-	99.25
420	95.18	91.02	-	98.05	-	93.54	-	-
480	95.48	92.08	-	98.20	-	94.68	-	-

Released amount of Hyd.HCl from matrix tablets are shown in Table 3. According to the results, tablets prepared with 50% (w/w) Carbopol 1342 (F 2) were found to be the most suitable formulation for Hyd.HCl followed by 40% Carbopol® 1342 (F 6) and 50% Carbopol® 834 (F 1). Hence F2 was considered as the best formula to be kinetically evaluate using a computer program (Ağabeyoğlu, 1984) (Table 4). The highest determination

coefficient and the best linear relation were observed with F2 in respect to RRSBW kinetics (Fig 1).

When the branched polymer is in contact with synthetic gastric liquid, it turns into a gel, because of the liquid transfer into the polymer. On the other hand, release of the drug is observed, which does not follow the classical kinetic equation, as the release are partially controlled by diffusion.

This study showed that the release mechanism in a matrix system comprising an insoluble hydrophobic and hydrophilic gel-forming parts, depends greatly on the wettability of the added drug. Furthermore, with wettable and water soluble drug, the matrix swells and release is mainly achieved by diffusion due to dissolution of the gel formed.

Table 4 Kinetic assessment of release data for formulation F 2

Kinetic Models

Hixon-Crowell (modified)	r^2	=	0.9294
	A	=	0.6465
	B	=	$1.0648.10^{-3}$
	AIC	=	-29.3803
	WSSD	=	0.0808
First order	r^2	=	0.9216
	Kr'	=	0.2798 h^{-1}
	AIC	=	-12.4224
	WSSD	=	0.4213
Zero order	r^2	=	0.7236
	Kro	=	4.5573 mg/h
	AIC	=	-0.4949
	WSSD	=	0.4946
RRSBW	r^2	=	0.9525
	$T_{463.2}$	=	128.0140 min.
	B	=	0.8014
	AIC	=	-32.8761
	WSSD	=	0.4191
Q square root of time	r^2	=	0.8344
	k	=	6.6410
	AIC	=	-19.1598
	WSSD	=	0.5369
Higuchi	r^2	=	0.9041
	Slope	=	1.4591

Özet

Bu çalışmanın amacı, antihipertansif ilaç olarak kullanılan hidralazin hidroklorür'ün uzatılmış etki gösteren matriks tabletini hazırlamaktır. Bunun için polimer olarak değişik konsantrasyonlarda karbomerler (Carbopols® 834, 934, 940 ve 1342), kaydırıcı olarak ise magnezyum stearat kullanılmış ve tabletler direkt basım tekniğine göre basılmıştır.

Tabletlerde etken madde, yükseklik-çap oranı, ağırlık sapması, ufalanma - aşınma kontrolü ve *in vitro* çözünme hızı tayinleri yapılmıştır. Etken madde miktar tayini spektrofotometrik yöntem ile, *in vitro* çözünme hızı testleri ise döner sepet yöntemi (USP XXII) ile yapılmış, zamana

Karşı etken maddenin yüzde salım profilleri çizilmiş ve çözünme hızı sonuçları kinetik açıdan incelenmiştir.

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Mechanism of Sustained-Action Medication

Theoretical Analysis of Rate of Release of Solid Drugs Dispersed in Solid Matrices

By T. HIGUCHI

Theoretically expected rates of release of solid drugs incorporated into solid matrices have been derived for several model systems. Mathematical relations have been obtained for cases (a) where the drug particles are dispersed in a homogeneous, uniform matrix which acts as the diffusional medium and (b) where the drug particles are incorporated in an essentially granular matrix and released by the leaching action of the penetrating solvent. Release from both planar surface and a sphere is considered. The unidimensional release rates are shown to follow our earlier equation derived for release from ointment bases. Release rates from spherical pellets by both model mechanisms are shown not to follow first-order relationships. The analyses suggest that for the latter system the time required to release 50 per cent of the drug would normally be expected to be approximately 10 per cent of that required to dissolve the last trace of the solid drug phase in the center of the pellet.

THE PRESENT COMMUNICATION is concerned with the results of a theoretical analysis of mathematical relationships governing the rate of release of solid drugs randomly dispersed in solid matrices. Systems of this type have been widely studied and utilized recently as bases for dosage forms which provide more or less continuous release of medicaments over relatively long periods (1). Both Wiegand and Taylor (2) and Wagner (3) showed that per cent released time data reported in the literature for many sustained-release preparations give linear pseudo (or apparent) first-order rates over the terminal portions of the data from about 0.5 hour to the time the test was completed. The present study is an effort in relating the rate of release of drugs from such systems to the pertinent physical constants based on simple laws of diffusion.

Two geometric systems have been considered: (a) unidirectional leaching or extraction from a simple planar surface, and (b) three dimensional leaching or extraction from a spherical pellet. This would correspond most closely to the release process from an insoluble tablet matrix or certain sustained-action pellets.

Two mechanisms of release from these systems have been treated. (a) Extraction of the medicament by a simple diffusional process through and from an enveloping, homogeneous matrix. The drug is presumed to go successively from the crystal surfaces into the uniform matrix and out into the bathing solvent which in turn acts as a perfect sink. (b) Leaching of the medicament by the bathing fluid which is able

to enter the drug-matrix phase through pores, cracks, and intergranular spaces. The drug is presumed to dissolve slowly into the permeating fluid phase and to diffuse from the system along the cracks and capillary channels filled with the extracting solvent. Intragranular diffusion is assumed, in this instance, to be insignificant. The two mechanisms are depicted schematically in Fig. 1.

It should be explicitly pointed out that the analyses reported here relate to these particular model systems, whereas the analyses of Wiegand and Taylor (2) and of Wagner (3) related to release data derived from formulated sustained-release or prolonged-action formulations. Actual dosage systems may be complicated by (a) simultaneous break-up of the matrix, (b) partial dissolution of the matrix substances, (c) one fraction of the dose being in a matrix form and the remainder of the dose being in a different, non-matrix and readily available form, and (d) drug on the surface being released more rapidly than drug in the matrix. Where such complications are absent, the treatments under *Theoretical Analysis* are believed to yield the correct relationships.

THEORETICAL ANALYSIS

Release from a Planar System Having a Homogeneous Matrix.—The amount of total drug released from such a system into a bathing medium acting essentially as a perfect sink would be determined by the relationship

$$Q = \sqrt{Dt(2A - C_s)C_s} \quad (\text{Eq. 1})$$

where Q = the amount of drug released after time t per unit exposed area, D = the diffusivity of the drug in the homogeneous matrix media, A = the total amount of drug present in the matrix per unit volume, and C_s = the solubility of the drug in the

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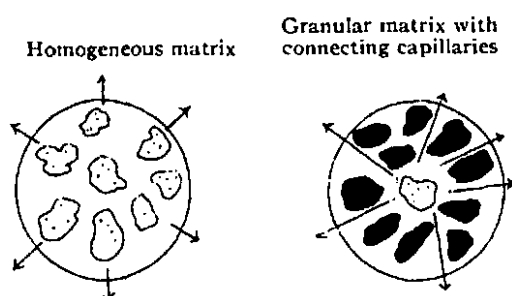


Fig. 1.—Two methods of drug release from the pellets.

matrix substance. We initially derived this equation for release from an ointment base containing finely dispersed drugs (4), but it is evident that it would be equally applicable for release from a sustained-action matrix of this type.

Release from a Planar System Having a Granular Matrix.—For the leaching type release mechanism occurring through diffusion movement utilizing intergranular openings, the above relation must be modified for the effective volume where diffusion can occur and the effective diffusional path. It can readily be seen for this system that

$$Q = \sqrt{\frac{D\epsilon}{\tau}} (2A - \epsilon C_s) C_s t \quad (\text{Eq. 2})$$

where Q = the amount of drug released after time t per unit exposed area, D = the diffusivity of the drug in the permeating fluid, τ = the tortuosity factor of the capillary system $\cong 3$, A = the total amount of drug present in the matrix per unit volume, C_s = the solubility of the drug in the permeating fluid, and ϵ = the porosity of the matrix.

The derivation of the above expression is essentially the same as Eq. 1, except that the cross-sectional area of the diffusional path must be reduced by the porosity factor ϵ , and the apparent solubility of the drug in the total system per unit volume must also be decreased by the same factor. The tortuosity factor, τ , is introduced to correct, in the same sense used in the classical Kozeny equation, for the lengthened diffusional path caused by the necessary lateral excursions.

For both equations the derivation (4) is based on the existence of a pseudo steady state condition during the release process and on the assumption that the drug particles are quite small relative to the average distance of diffusion and are uniformly distributed in the matrix. The equations would be essentially valid for systems in which A is greater than C_s or ϵC_s by a factor of three or four. Of course, if $A < C_s$ or ϵC_s , the drug would no longer be present as a solid and a different equation would apply.

Since the porosity factor in Eq. 2 refers, of course, to the porosity of the leached portion of the pellet, it differs from the initial porosity of the initially formed matrix. The difference would correspond directly to the volume of free space previously occupied by the extracted component or components. Thus

$$\epsilon = \epsilon_0 + KA$$

for systems where the drug is the only extractable

component, K being introduced to convert A to its corresponding volume fraction. K is equal to the specific volume of the drug = $1/(\text{density of the drug})$ if A is expressed in terms of grams of drug per milliliter. For those instances where the initial porosity, ϵ_0 , is very small or where the fraction of the matrix volume occupied by the drug is relatively large $\epsilon \cong KA$ and Eq. 2 reduces to

$$Q = A \sqrt{DK/\tau(2 - KC_s)C_s}. \quad \text{Thus for these systems it would appear that the fraction of the drug released at anytime is essentially independent of } A.$$

Release from a Spherical Pellet Having a Homogeneous Matrix.—Any attempt to derive an exact solution for a system of this type is, of course,

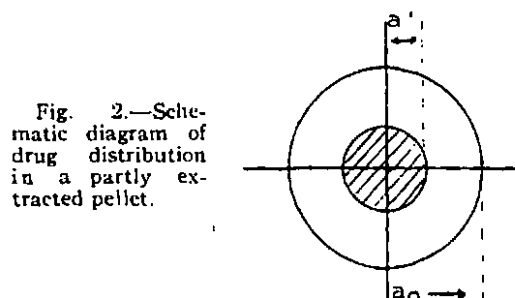


Fig. 2.—Schematic diagram of drug distribution in a partly extracted pellet.

impossible since it would require an exact coordinate description of the distribution pattern of the dispersed particles. A reasonably accurate and useful mathematical solution can be based on the same assumption which permitted solution of the two-dimensional system. We can again assume for the case $A \gg C_s$ that a pseudo steady state condition would exist during the leaching or extraction process and that a sharp front will be formed between the partly leached or extracted part of the sphere and the untouched portion. This state of affairs is shown in Fig. 2 where a_0 = the radius of the whole pellet, a' = the radius of that part still unextracted, and a = the polar radius of any region under consideration, and the remaining symbols are the same as before.

It is evident in such a system that the concentration gradient is essentially zero for $a < a'$. The concentration in the region between a' and a_0 will be a function of a and is assumed to be that fixed by Fick's first law.

Under pseudo steady state conditions as described above the total amount of material, SdQ , being released per unit time, dt , will be given by Fick's first law as

$$\frac{SdQ}{dt} = -4\pi a^2 D \frac{dC}{da} \quad (\text{Eq. 3})$$

where S is the diffusional area for $a' \leq a \leq a_0$. Integrating this from a to a_0 we obtain

$$\left(\frac{SdQ}{dt}\right)_t \left(\frac{1}{a} - \frac{1}{a_0}\right) = 4\pi DC_a$$

where C_a = concentration at a or

$$\left(\frac{SdQ}{dt}\right)_t = \frac{4\pi DC_a}{\left(\frac{1}{a} - \frac{1}{a_0}\right)} = \frac{4\pi DC_s}{\left(\frac{1}{a'} - \frac{1}{a_0}\right)} \quad (\text{Eq. 4})$$

since $C_a = C_s$ at $a = a'$ and $C_a = 0$ at $a = a_0$ and

$$C_a = C_s \frac{a'}{a} \frac{(a_s - a)}{(a_s - a')} \quad (\text{Eq. 5})$$

an expression which relates concentration to a in the region $a' < a < a_s$.

It is apparent that the total amount of the drug contained by the pellet at time t is the sum of that in the unleached portion ($a < a'$) and that in the region no longer saturated with the drug ($a' < a < a_s$). Or the total residual amount of drug equals

$$\begin{aligned} & \frac{4}{3} a'^3 \pi A + \int_{a'}^{a_s} 4 \pi a^2 C_a da \quad (\text{Eq. 6}) \\ &= \frac{4}{3} \pi a'^3 A + \int_{a'}^{a_s} 4 \pi a^2 C_s \frac{a'}{a} \frac{(a_s - a)}{(a_s - a')} da \\ &= \frac{4}{3} \pi a'^3 A + \frac{4}{6} \pi C_s \frac{a'}{a_s - a'} (a_s^3 - 3a_s a'^2 + 2a'^3) \\ &= 4\pi \left[\frac{a'^3}{3} A + \frac{C_s}{6} a' (a_s^3 + a' a_s - 2a'^2) \right] \quad (\text{Eq. 7}) \end{aligned}$$

The change in the residual drug concentration corresponding to a change $-da'$ would then be

$$= 4\pi \left[A a'^2 + \frac{C_s}{6} (a_s^3 + 2a' a_s - 6a'^2) \right] (-da') \quad (\text{Eq. 8})$$

This should in turn be equal but opposite in sign to the total flux over the period involved

$$\begin{aligned} \left(\frac{SdQ}{dt} \right) dt &= \frac{4\pi DC_s}{\left(\frac{1}{a'} - \frac{1}{a_s} \right)} dt = \\ 4\pi \left[A a'^2 + \frac{C_s}{6} (a_s^3 + 2a' a_s - 6a'^2) \right] da' \\ DC_s dt &= \left(\frac{a_s - a'}{a a'} \right) \left[A a'^2 + \frac{C_s}{6} (a_s^3 + \right. \\ & \quad \left. 2a' a_s - 6a'^2) \right] da' \quad (\text{Eq. 9}) \end{aligned}$$

Integrating from a_s to a' we obtain

$$A(a_s^3 + 2a'^3 - 3a_s a'^2) + C_s \left(4a'^2 a_s + a_s^3 \ln \frac{a_s}{a'} - a_s^3 - a_s^2 a' - 2a'^3 \right) = 6DC_s a_s t \quad (\text{Eq. 10})$$

For $C_s \ll A$ the above reduces to

$$a_s^3 - 3a'^2 a_s + 2a'^3 \approx \frac{6a_s}{A} DC_s t \quad (\text{Eq. 11})$$

Equation 10 represents a general solution to the proposed problem since it permits determination of a' as a function of time, t , if the constants of the system, a_s , A , C_s , and D are known. Since the residual amount of drug at any time is already expressed by Eq. 7 in terms again of a' , the total amount of drug released as a function of time can be readily calculated. Since any attempt to convert Eq. 10 into an explicit solution for a' as a function of t would result in a cumbersome relationship, it is more feasible in practice to obtain t as a function of a' and the amount of release also as a function

of a' from Eq. 7 and then correlate the two dependent variables.

A general solution to Eq. 11 can be obtained in this implicit manner. For this it is more convenient to transform Eq. 11 into a dimensionless relationship

$$1 - 3\left(\frac{a'}{a_s}\right)^2 + 2\left(\frac{a'}{a_s}\right)^3 = \frac{6DC_s}{Aa_s^3} t = Bt \quad (\text{Eq. 12})$$

In this form the left hand expression in terms of a'/a_s is dimensionless and is independent of any units of measure employed. The factor, $B = 6DC_s/Aa_s^3$ is dimensionless except for time and can be calculated from the constants of the system. Since the fraction of drug remaining in the pellet for this system where $A \gg C_s$ would be

$$\text{Residual fraction of drug in pellet} = \left(\frac{a'}{a_s}\right)^3 \quad (\text{Eq. 13})$$

we can readily prepare a plot of the residual fraction remaining in the pellet as a function of relative time. Actual time unit can be substituted in real systems where the constants comprising B are determinable. The results are shown for different (a'/a_s) values in Table I and are plotted in Fig. 3. It is evident

TABLE I.—CALCULATION OF RELEASE RATE FROM SPHERICAL PELLET $A \gg C_s$

$\left(\frac{a'}{a_s}\right)$	Time Scale		Drug Remaining (%) in Pellet $\left(\frac{a'}{a_s}\right)^3 \times 100$
	$\left(\frac{a'}{a_s}\right)$	Bt	
1.00	0.0000		100.0
0.990	0.00030		97.03
0.980	0.001184		94.12
0.950	0.00725		85.70
0.90	0.02800		72.90
0.80	0.1040		51.20
0.70	0.2160		34.30
0.60	0.3520		21.60
0.50	0.5000		12.90
0.40	0.8480		6.40
0.30	0.7840		2.70
0.20	0.8960		0.800
0.10	0.9720		0.100
0.00	1.000		0.000

* Equation 11.

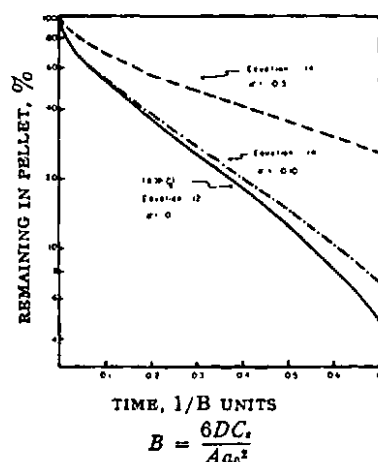


Fig. 3.—Influence of $\alpha = C_s/A$ values on rate of release.

from the table that the physical significance of B is that for the case $A \gg C_s$ its reciprocal corresponds to that time when the last trace of the solid drug dissolves into the matrix. Under these conditions the pellet can be considered to be totally exhausted. Also from the table it is apparent that the drug is very rapidly released at the beginning, approximately 50% of it being released at $t = 0.1 \times 1/B$.

The values shown in Table I have been plotted in Fig. 3 in the usual semilogarithmic fashion to show that the release rate predicted by this mechanism departs significantly from the first-order behavior. Aside from what appears to be a large initial surge of release, however, the plot yields a surprisingly linear relationship over a relatively wide range. This seems to arise from the fact that it is actually sigmoidal in nature and the apparent linear relationship observed (2, 3) may for some system be coincidental and for others be because of different overall mechanism.

In Fig. 4 the fraction of drug released from a sphere calculated from Eq. 11 is compared to that expected from Eq. 1 on the basis that the total exposed surface is $4\pi a_0^2$. The plot has been made as a function of $(Bt)^{1/2}$ since this affords a linear relationship for the two-dimensional system. It is evident that at the beginning the two equations predict a similar extent of release, as expected, since a plane of the same area would be a good approximation for the sphere in this phase. It is only beyond 50% release that significant deviation is evident between the two equations.

The more general Eq. 10 which takes into account the amount of drug remaining in the partly extracted region can be solved in an identical manner based on Eqs. 7 and 10. Setting $C_s = \alpha A$ and rearranging Eq. 10 we obtain

$$1 + 2 \left(\frac{a'}{a_0} \right)^2 - 3 \left(\frac{a'}{a_0} \right)^3 + \alpha \left[4 \left(\frac{a'}{a_0} \right)^2 + \ln \frac{a_0}{a'} - \frac{a'}{a_0} - 2 \left(\frac{a'}{a_0} \right)^3 - 1 \right] = \frac{6DC_s t}{Aa_0^3}$$

or

$$1 - \alpha + 2(1 - \alpha) \left(\frac{a'}{a_0} \right)^2 - (3 - 4\alpha) \left(\frac{a'}{a_0} \right)^3 - \alpha \left(\frac{a'}{a_0} \right) + \alpha \ln \frac{a_0}{a'} = \frac{6DKC_s t}{Aa_0^3} \quad (\text{Eq. 14})$$

Release behavior predicted by this equation for several values of α are also shown in Fig. 3. Residual fraction in these instances based on Eq. 7 would be

$$\text{Residual Fraction} = \left(\frac{a'}{a_0} \right)^3 + \frac{\alpha}{2} \left[\left(\frac{a'}{a_0} \right) + \left(\frac{a'}{a_0} \right)^2 - 2 \left(\frac{a'}{a_0} \right)^3 \right] \quad (\text{Eq. 15})$$

Since in most real systems α is usually very small, any term containing it as a factor can normally be ignored.

Plots shown in Fig. 3 are, in a sense, universal relationships in that they permit estimation of the release behavior for all systems for given α values. They indicate that the relative release rates (*i.e.*, the fraction released per unit time) from pellets of this type would be inversely proportional to the

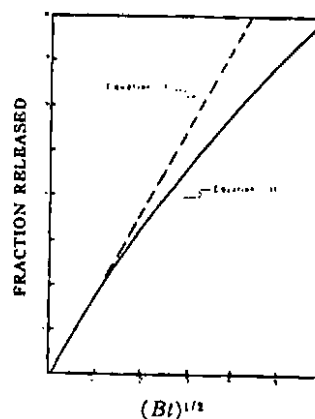


Fig. 4.—Comparison of Eqs. 1 and 11.

square of the radius of the pellet and the drug concentration but directly proportional to the solubility and the diffusivity of the drug. The plot shown for $\alpha = 0.5$ is less valid than the others since the assumed pseudo steady state condition requires A to be significantly larger than C_s . Actually α must be considerably smaller for the spherical three-dimensional system for the steady state situation to exist than for the planar system. This is especially true for higher states of depletion.

Release by Leaching from Granular Spherical Pellet.—The solution to the rate of leaching by external solvent (*e.g.*, gastric fluid) of solid drugs uniformly dispersed in granular spherical pellets can be developed exactly as in the preceding case. It is evident that the dimensionless expression corresponding to Eq. 14 will take the form

$$(1 - \alpha) + 2 \left(\frac{a'}{a_0} \right)^2 (1 - \alpha) - \left(\frac{a'}{a_0} \right)^3 (3 - 4\alpha) - \alpha \left(\frac{a'}{a_0} \right) + \alpha \ln \left(\frac{a_0}{a'} \right) = \frac{6D\epsilon C_s t}{\tau A a_0^3} \quad (\text{Eq. 16})$$

where $\alpha = \epsilon C_s / A$ and $\epsilon = \epsilon_0 + KA$, and the remaining symbols the same meanings as before with D being the diffusivity in the solvent. If as in the two-dimensional case we take the initial porosity, ϵ_0 , as being negligible we obtain

$$(1 - \alpha) + 2 \left(\frac{a'}{a_0} \right)^2 (1 - \alpha) - \left(\frac{a'}{a_0} \right)^3 (3 - 4\alpha) - \alpha \left(\frac{a'}{a_0} \right) + \alpha \ln \left(\frac{a_0}{a'} \right) = \frac{6DKC_s t}{\tau a_0^3}$$

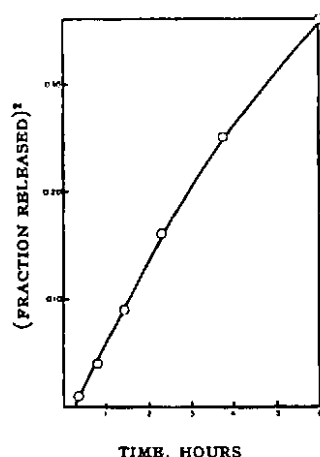
For $\alpha \ll 1$, this reduces to

$$1 + 2 \left(\frac{a'}{a_0} \right)^2 - 3 \left(\frac{a'}{a_0} \right)^3 + \frac{6DKC_s t}{\tau a_0^3} \quad (\text{Eq. 17})$$

where the residual fraction = $(a'/a_0)^3$.

In these analyses it has been tacitly assumed that the time of extraction begins with the slightly porous pellet already permeated by the extracting solution. Since in practice such pellets (or "cores") will be taken dry there will be a short lag time corresponding to that required to wet the interior of the matrix. This time, however, should normally be relatively small compared to the duration of action of such medications.

Fig. 5.—Comparison of Simoons' experimental data (5) with Eq. 17.



GENERAL DISCUSSION

The several solutions to the release behavior of the systems considered are believed to be essentially exact for the models employed. Experimental data available appear to substantiate these analyses (5).

In real systems, however, a number of other factors may come into play which may modify the total behavior. The models employed assume that the systems are neither surface coated, nor that their matrices undergo significant alteration in the presence of moisture. Since in real systems these play varying roles in modifying the release pattern of sustained-action dosage forms, any attempt to apply these equations must be made with this in mind.

Other serious deviations from the derived relation-

ship may occur for systems which tend to differ significantly from the adopted models. For example, for pellets containing a relatively high percentage of drugs the leaching process would tend to weaken the matrix structure and produce erosion. This may play a significant role in altering the observed real rate. Another effect which is not considered in these treatments is the influence of solvent flow induced within the pellets by external agitation. This effect, however, will be important only with pellets of relatively high porosity.

In Fig. 5 data reported by Simoons in his interesting paper on experimental measurements of release rate of sustained-action medication (6) for relatively insoluble drug hard compressed (his Fig. 15) are plotted in the form of square of fraction released against extraction time. The extraction data show a short lag time probably corresponding to that required to wet the pellets. This is followed by a release pattern closely matching that predicted by Eq. 17, the smooth line representing the theoretical values and the points, the experimentally observed values reported by Simoons. The total theoretical curve was based on experimentally observed time for 50% release and the initial lag time.

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Dielectric Constants of Complex Pharmaceutical Solvent Systems I

Water-Ethanol-Glycerin and Water-Ethanol-Propylene Glycol

By D. L. SORBY, R. G. BITTER, and J. G. WEBB

Dielectric constants of water-ethanol-glycerin and water-ethanol-propylene glycol systems have been experimentally determined. The measured values were found to differ from values calculated according to simplification of the Onsager-Kirkwood equation, regardless of whether composition of the various solutions was expressed on the basis of weight percentage or volume percentage. Dielectric constant values presented in this paper are recommended for precise adjustment of solvent polarity in formulation work and data are presented to be of maximum use in this respect.

MOORE (1) HAS PRESENTED a method wherein manipulation of solvent dielectric constant is utilized to produce dissolution of a solute at a desired concentration and to blend pharmaceutical solvents to a predetermined degree of polarity. In this method, certain simplifying

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assumptions are made, one being that the dielectric constant of a complex solvent mixture may be calculated to a good approximation by taking the sum of the products of volume composition and dielectric constant for each individual component in the mixture. This method of calculating dielectric constants of complex mixtures is based on a simplification of the Onsager-Kirkwood