High-Pressure Liquid Chromatographic Determination of Salicylsalicylic Acid, Aspirin, and Salicylic Acid in Human Plasma and Urine

L. I. HARRISON ˣ, M. L. FUNK, and R. E. OBER

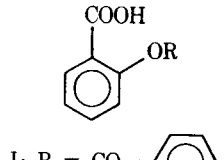
Received March 10, 1980, from the *Drug Metabolism Department, Riker Laboratories, Inc., Subsidiary of 3M, 3M Center, St. Paul, MN 55144.* Accepted for publication May 2, 1980.

Abstract □ A high-pressure liquid chromatographic method was developed for the separation and quantitation of salicylsalicylic acid (I), aspirin (II), and salicylic acid (III) in human plasma and urine. The method for plasma involves the selective extraction of I–III and an internal standard, α-phenylcinnamic acid, into methylene chloride from acidified plasma, followed by evaporation of the organic phase and dissolution of the residue in methanol. A 25-μl aliquot is analyzed on a reversed-phase column with UV detection. Urine is treated similarly with hexane as the extracting solvent. If 300 nm is used as the monitoring wavelength, the assays of I and III are linear over the concentration range of 1–150 μg/ml in both plasma and urine. If 280 nm is used as the monitoring wavelength, II can be quantitated along with I and III; however, detector sensitivities of I and III are two to three times greater at 300 nm. The accuracy and precision of the methods for I–III are adequate for clinical pharmacokinetic studies. Following therapeutic doses of I in humans, the method was applied successfully to the determination of I and III in plasma and urine.

Keyphrases □ Aspirin—high-pressure liquid chromatographic analysis with salicylsalicylic acid and salicylic acid in human plasma and urine □ Salicylsalicylic acid—high-pressure liquid chromatographic analysis with aspirin and salicylic acid in human plasma and urine □ Salicylic acid—high-pressure liquid chromatographic analysis with salicylsalicylic acid and aspirin in human plasma and urine □ High-pressure liquid chromatography—analysis, salicylsalicylic acid, aspirin, and salicylic acid in human plasma and urine

Salicylsalicylic acid (I) was developed as a salicylate alternative for aspirin (II) and sodium salicylate. Although I was available as early as 1920, its usefulness in salicylate therapy has been explored only recently (1). Double-blind clinical trials have shown that I causes significantly less GI bleeding and gastric erosions at anti-inflammatory doses than does II (2–6). The therapeutic response of arthritic patients to I appears to be equivalent to that of II (6–11). Thus, I appears to be an important salicylate derivative and offers efficacy with diminished GI distress.

Compound I is hydrolyzed in the body to give salicylic acid (III). Metabolic studies of I measured only levels of III following single and multiple doses of I (5, 11–16). Studies of plasma and urine levels of I used the difference between levels of III before and after hydrolysis as an estimate of the concentrations of I (11, 16). A high-pressure liquid chromatographic (HPLC) assay was described recently for monitoring I as an impurity in aspirin tablets (17). The present report describes a specific and sensitive HPLC assay for the quantitation of I–III in biological samples.



I: R = CO–  OH

II: R = $COCH_3$

III: R = H

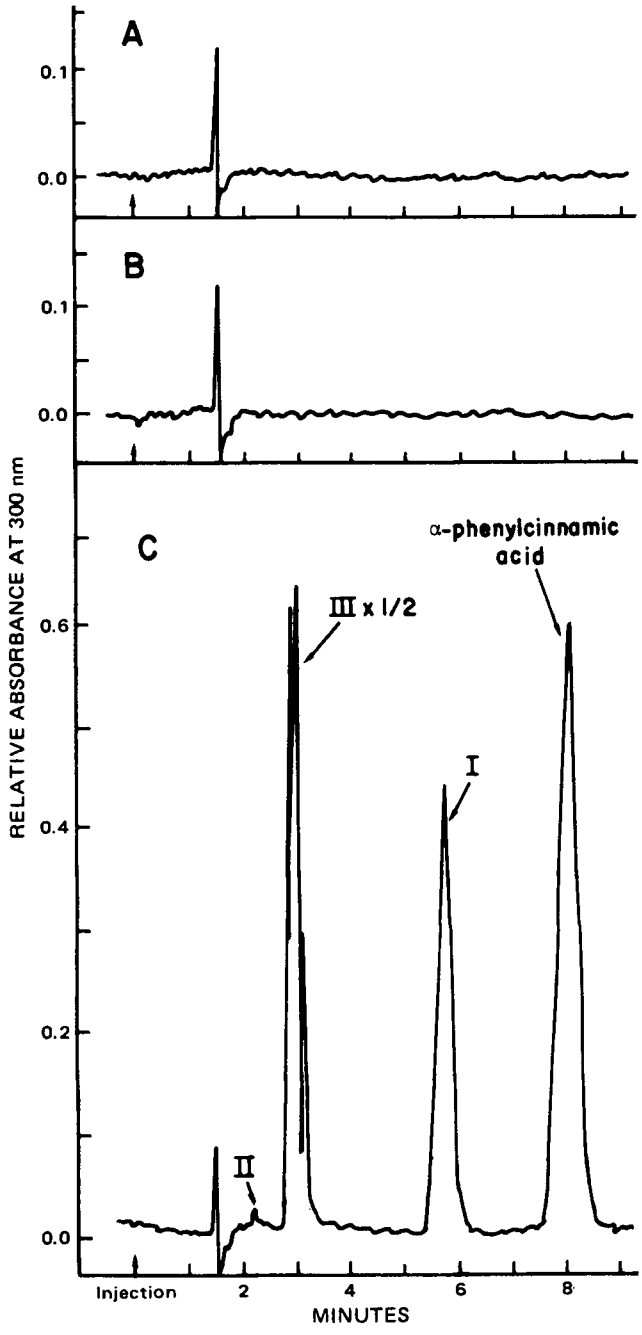


Figure 1—*HPLC assay of I and III at 300 nm. Key: A, drug-free human plasma; B, drug-free human urine; and C, plasma containing 20 μg of I–III and α-phenylcinnamic acid/ml.*

0022-3549/80/1100-1268$01.00/0

Table I—Accuracy and Precision of the HPLC Analysis of Salicylsalicylic Acid and Salicylic Acid in Human Plasma at 300 nm

Drug Added to Plasma, μg/ml	Salicylsalicylic Acid		Salicylic Acid	
	Detected, μg/ml	Difference, μg/ml	Detected, μg/ml	Difference, μg/ml
5.0	5.2	0.2	6.2	1.2
	5.1	0.1	6.2	1.2
	5.0	0.0	6.0	1.0
	5.5	0.5	6.1	1.1
	5.3	0.3	6.0	1.0
	5.2 ± 0.2[a]	0.2 *ME*[b]	6.1 ± 0.1[a]	1.1 *ME*
	4% *CV*[c]	4% *RE*[d]	2% *CV*	18% *RE*
20.0	20.7	0.7	20.7	0.7
	20.0	0.0	21.4	1.4
	20.8	0.8	21.2	1.2
	20.8	0.8	20.3	0.3
	20.9	0.9	20.1	0.1
	20.6 ± 0.4[a]	0.6 *ME*	20.7 ± 0.6[a]	0.7 *ME*
	2% *CV*	3% *RE*	3% *CV*	3% *RE*
50.0	48.1	−1.9	47.7	−2.3
	50.8	0.8	47.8	−2.2
	49.3	−0.7	52.4	2.4
	49.0	−1.0	48.9	−1.1
	52.0	2.0	51.2	1.2
	49.8 ± 1.6[a]	−0.2 *ME*	49.6 ± 2.1[a]	−0.4 *ME*
	3% *CV*	−0.4% *RE*	4% *CV*	−0.8% *RE*

[a] Mean ± *SD*. [b] *ME* = mean error. [c] *CV* = coefficient of variation × 100. [d] *RE* = relative error.

Table II—Accuracy and Precision of the HPLC Analysis of Salicylsalicylic Acid and Salicylic Acid in Human Urine at 300 nm

Drug Added to Urine, μg/ml	Salicylsalicylic Acid		Salicylic Acid	
	Detected, μg/ml	Difference, μg/ml	Detected, μg/ml	Difference, μg/ml
5.0	5.1	0.1	5.5	0.5
	5.9	0.9	5.8	0.8
	5.6	0.6	6.0	1.0
	4.9	−0.1	5.4	0.4
	5.4 ± 0.46[a]	0.4 *ME*[b]	5.7 ± 0.28[a]	0.7 *ME*
	8.5% *CV*[c]	7% *RE*[d]	4.9% *CV*	12% *RE*
10.0	9.8	−0.2	8.6	−1.4
	9.6	−0.4	9.7	−0.3
	9.2	−0.8	9.3	−0.7
	9.7	−0.3	9.3	−0.7
	9.6 ± 0.26[a]	−0.4 *ME*	9.2 ± 0.46[a]	−0.8 *ME*
	2.7% *CV*	−4% *RE*	5.0% *CV*	−9% *RE*

[a-d] See Table I.

EXPERIMENTAL

Materials—All solvents were glass distilled and suitable for liquid chromatographic analyses[1], and all reagents were analytical grade. Analytically pure standards of I[2]–III and α-phenylcinnamic acid[3] were used. Aqueous solutions were prepared in distilled water. Drug-free plasma and urine were obtained from normal human volunteers.

A high-pressure liquid chromatograph[4] equipped with a septumless injector[5] and a variable-wavelength UV detector[6] were used. The detector was operated at either 0.02 or 0.04 aufs. The mobile phase of methanol–1% acetic acid (60:40 v/v) was pumped at 2.0 ml/min (2800 psi) through a stainless steel column (30 cm × 4 mm i.d.) packed with a high-efficiency, reversed-phase packing[7].

Methods—α-Phenylcinnamic acid served as the internal standard. To a 0.5-ml aliquot of plasma were added 0.9 ml of 0.27 *N* HCl, 10 μg of α-phenylcinnamic acid (100 μl of a 0.1-mg/ml solution in methanol), and 10 ml of methylene chloride. The tubes were shaken for 15 min on a mechanical shaker at 125 cycles/min and centrifuged for 5 min at 750×*g*.

[1] Burdick & Jackson Laboratories, Muskegon, Mich.
[2] Riker Laboratories, Northridge, Calif.
[3] Aldrich Chemical Co., Milwaukee, Wis.
[4] Model 6000, Waters Associates, Milford, Mass.
[5] Model U6K, Waters Associates, Milford, Mass.
[6] Model SF 770, Schoeffel Instrument Corp., Waterford, N.J.
[7] μBondapak C_{18}, Waters Associates, Milford, Mass.

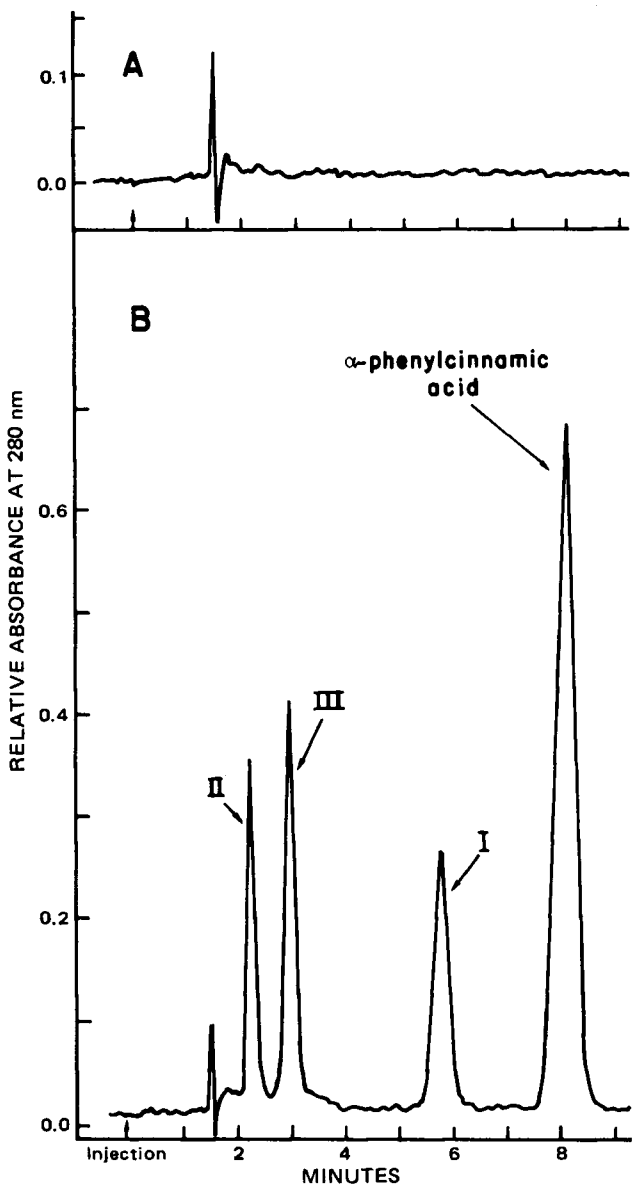


Figure 2—*HPLC assay of I–III at 280 nm. Key: A, drug-free human plasma; and B, plasma containing 20 μg of I–III and α-phenylcinnamic acid/ml.*

The methylene chloride phase was separated and evaporated to dryness[8]. The residue was dissolved in 0.5 ml of methanol, and 25 μl was injected into the chromatograph.

A similar procedure was used for the urine extraction, except that 2-ml urine aliquots were extracted with 10 ml of hexane. A solvent less polar than methylene chloride was required for the urine extractions to eliminate interfering substances.

Plasma and urine standards were prepared by adding I and III (and in some cases II) dissolved in 100 μl of methanol to aliquots of drug-free human plasma and urine. UV absorbance was measured at 300 nm. Peak height ratios were used to determine concentrations.

RESULTS AND DISCUSSION

Reversed-phase HPLC was an effective method to quantitate I and III simultaneously in human plasma and urine. Figure 1 shows a representative chromatogram. Baseline separation between I, III, and α-phenylcinnamic acid (the internal standard) was achieved. No interference by endogenous materials from either drug-free human plasma or urine was seen (Fig. 1). Under the experimental conditions, the elution

[8] Evapo-Mix, Buchler Instruments, Fort Lee, N.J.

Table III—Accuracy and Precision of the HPLC Analysis of Salicylsalicylic Acid, Aspirin, and Salicylic Acid in Human Plasma at 280 nm

Drug Added to Plasma, μg/ml	Salicylsalicylic Acid		Aspirin		Salicylic Acid	
	Detected, μg/ml	Difference, μg/ml	Detected, μg/ml	Difference, μg/ml	Detected, μg/ml	Difference, μg/ml
5.0	5.9	0.9	5.1	0.1	5.6	0.4
	5.6	0.6	5.2	0.2	6.1	1.1
	5.8	0.8	5.2	0.2	5.9	0.9
	6.3	1.3	5.2	0.2	6.1	1.1
	5.9	0.9	4.4	−0.6	6.4	1.4
	5.9 ± 0.3[a]	0.9 *ME*[b]	5.0 ± 0.3[a]	0.02 *ME*	6.0 ± 0.3[a]	1.0 *ME*
	4% *CV*[c]	15% *RE*[d]	6% *CV*	0.4% *RE*	5% *CV*	17% *RE*
20.0	20.2	0.2	20.3	0.3	20.2	0.2
	19.9	−0.1	19.8	−0.2	21.2	1.2
	20.8	0.8	20.6	0.6	20.6	0.6
	20.9	0.9	20.4	0.4	20.6	0.6
	20.5	0.5	20.1	0.1	19.7	−0.3
	20.5 ± 0.4[a]	0.5 *ME*	20.2 ± 0.3[a]	0.2 *ME*	20.5 ± 0.6[a]	0.5 *ME*
	2% *CV*	2% *RE*	1% *CV*	1% *RE*	3% *CV*	2% *RE*
50.0	48.1	−1.9	49.6	0.4	48.4	−1.6
	50.5	0.5	49.4	0.6	48.1	−1.9
	49.3	−0.7	50.0	0.0	52.0	2.0
	49.3	−0.7	49.7	−0.7	49.2	−0.8
	52.0	2.0	50.7	0.7	51.1	1.1
	49.8 ± 1.5[a]	−0.2 *ME*	49.9 ± 0.5[a]	−0.2 *ME*	49.8 ± 1.7[a]	−0.2 *ME*
	3% *CV*	−0.4% *RE*	1% *CV*	0.4% *RE*	3% *CV*	−0.4% *RE*

[a–d] See Table I.

volumes for I, III, and the internal standard were 11.5 (5.8 min), 6.0 (3.0 min), and 16 (8.0 min) ml, respectively.

The assay of I and III was linear over the concentration range of 1–150 μg/ml in both plasma and urine. The minimum detectable level of I and III was 1 μg/ml. However, this sensitivity could be increased by adding <500 μl of methanol at the final extraction step. The accuracy and precision of the method were tested at three concentrations of I and III in plasma. Both compounds were present in all plasma samples; five samples of each concentration were assayed on the same day (Table I).

The coefficient of variation was ≤4% for all three levels of I and III. Thus, good precision was demonstrated. The accuracy of the method is indicated by the mean error between the detected and theoretical values. The mean error was small in most cases, except for III at 5 μg/ml. Similar accuracy and precision were seen with urine samples (Table II).

Compound II was quantitated along with I and III by changing the wavelength to 280 nm (Fig. 2). Compound II had weak UV absorbance at 300 nm (Fig. 1) and could not be quantitated at that wavelength. The elution volume for II was 4.5 ml (2.3 min). Since the elution volumes for I, III, and the internal standard were unchanged, the analysis time remained at 9 min/sample. The assay at 280 nm for II (and also for I and III) was linear over the concentration range of 2–100 μg/ml. The accuracy and precision of the method at 280 nm were tested at three concentrations of I–III in plasma and were similar to those observed at 300 nm (Table III).

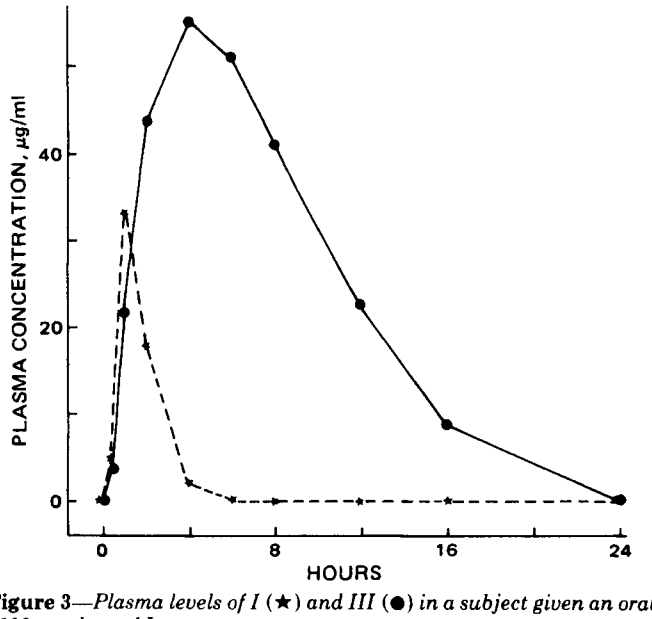


Figure 3—*Plasma levels of I (★) and III (●) in a subject given an oral 1000-mg dose of I.*

For maximum sensitivity, I and III should be quantitated at 300 nm and II should be quantitated at 280 nm. As shown in Fig. 2, the 280-nm wavelength can be used to quantitate I–III simultaneously in plasma, although the absorbances of I and III at 280 nm under the present experimental conditions were only 65 and 35%, respectively, of the absorbances at 300 nm.

The HPLC method was used in the analysis of plasma and urine samples from a 10-subject absorption study of I. The healthy male volunteers were given a single, oral, 1000-mg dose of I as two tablets[9]. Heparinized blood samples were taken immediately before dosing and at 0.5, 1, 2, 4, 6, 8, 12, 16, and 24 hr postdosing. The plasma levels of I and III were determined by the HPLC method at 300 nm and are plotted for a representative subject in Fig. 3. A peak plasma level for I of 33.4 μg/ml was reached in this subject at 1 hr postdosing, and the decrease in plasma levels followed a monoexponential decline with an estimated plasma half-life for I of 0.8 hr. The *in vivo* hydrolysis of I to III resulted in a peak plasma level of III of 55.2 μg/ml at 4 hr; the plasma half-life of III was 3.5 hr. The 24-hr urinary excretions of I and III in this subject were 10.0 and 29.8 mg, respectively.

During this absorption study, analytical results showed that the assay yielded only minimal day-to-day and operator-to-operator variations. Eleven plasma standard curves were run for each compound over a 2-month period. The coefficient of correlation of each plasma standard curve was 0.997 or better for I; it was 0.994 or better, with one exception (0.989), for III. Zero was contained within the standard error of the intercept. Linear regression of a line forced through the origin for the mean standard curve gave slopes of 0.035 ± 0.0031 (mean ± *SD*) for I and 0.084 ± 0.0062 for III. Comparable agreement was obtained for four urine standard curves.

Several multiple-dose studies at anti-inflammatory doses have shown that the assay is more than adequate to determine plasma concentrations of I in humans. However, simultaneous quantitation of III may require sample dilution to keep the level of III below 150 μg/ml. The HPLC assay requires 9 min/sample. Approximately 30 samples can be extracted and analyzed daily.

REFERENCES[10]

(1) W. W. Downie and V. Wright, *Practitioner*, **219**, 465 (1977).

[9] Disalcid, Riker Laboratories, Northridge, Calif.

[10] *Note added in proof:* Another HPLC assay for the quantitation of I and III in plasma was published recently (18).

(2) J. R. Leonards, *J. Lab. Clin. Med.*, **74**, 911 (1969).
(3) D. Edmar, *Acta Radiol.*, **11**, 57 (1971).
(4) S. Thune, *Nord. Med.*, **79**, 352 (1968).
(5) A. Cohen, *J. Clin. Pharmacol.*, **19**, 242 (1979).
(6) S. P. Liyanage and P. K. Tambar, *Curr. Med. Res. Opinion*, **5**, 450 (1978).
(7) L. J. Denson and W. R. Thompson, *J. Med. Soc. N.J.*, **85**, 314 (1960).
(8) L. Paris and H. Newfield, *Clin. Med.*, **70**, 2029 (1963).
(9) R. G. Regalado, *Curr. Med. Res. Opinion*, **5**, 454 (1978).
(10) O. N. Re, *J. Int. Med. Res.*, **7**, 90 (1979).
(11) P. Nordqvist, presented at the Congress of the Swedish Medical Society, Stockholm, Sweden, Nov. 26, 1964.
(12) P. Nordqvist, J. G. L. Harthon, and R. Karlsson, *Nord. Med.*, **74**, 1074 (1965).
(13) A. Cohen, *Curr. Ther. Res.*, **23**, 772 (1978).
(14) I. W. French and C. A. Mildon, *Clin. Ther.*, **1**, 353 (1978).
(15) I. Santillan, *Curr. Ther. Res.*, **23**, 345 (1978).
(16) P. J. Hanzlik and N. E. Presho, *J. Pharmacol. Exp. Ther.*, **26**, 61 (1926).
(17) J. C. Reepmeyer and R. D. Kirchhoefer, *J. Pharm. Sci.*, **68**, 1167 (1979).
(18) M. M. Lubran, S. N. Steen, and R. L. Smith, *Ann. Clin. Lab. Sci.*, **9**, 501 (1979).

Preparation, Characterization, and Stability of New Prostaglandin E_2 Gel for Local Administration

A. S. HARRIS *x, A. KIRSTEIN-PEDERSEN ‡, P. STENBERG §, U. ULMSTEN ¶, and L. WINGERUP ¶

Received March 18, 1980 from **Perstorp AB, Box 5000, S-28400 Perstorp, Sweden*, the ‡*Department of Pharmacology, University of Aarhus, DK-8000 Aarhus C, Denmark*, and the §*Hospital Pharmacy and* ¶*Departments of Obstetrics and Gynecology, Malmö General Hospital, S-214-01 Malmö, Sweden.* Accepted for publication May 2, 1980.

Abstract □ A new gel delivery system for the local application of prostaglandin E_2 consists of drug incorporated in the matrix of a cross-linked starch polymer. The properties of the starch powder provide a stabilizing milieu for the labile prostaglandin E_2 and, by addition of saline, a ready-to-use gel for immediate local administration. The gel offers advantages over existing preparations in terms of chemical and microbiological stability, homogeneity, and dosage safety. This report outlines the pharmaceutical aspects involved in the development of the delivery system.

Keyphrases □ Prostaglandin E_2—preparation, characterization, and stability of gel delivery system □ Drug delivery systems—prostaglandin E_2 gel, preparation, characterization, and stability

The discovery, isolation, and characterization of the various prostaglandins have created substantial interest in their physiological role and therapeutic applications (1). Their use in human reproduction (2) clearly demonstrates their clinical value. However, the broad spectrum of pharmacological responses and the general instability of the prostaglandins have made it difficult to achieve specific therapeutic aims without significant side effects.

BACKGROUND

In the E series, for example, the cervical ripening effect of prostaglandin E_2 first was observed in early studies using intravenous infusion prior to therapeutic abortion or induction of labor in patients at term (3). The large dose required to achieve these effects was associated with frequent systemic side effects such as vomiting and diarrhea (4). The most recent phase in such use of prostaglandin E_2 has been a low dose local (intracervical or vaginal) administration in a viscous gel or a lipid-based pessary (5, 6).

The prostaglandin E_2 regimen is simple and highly acceptable to patients, causing no significant side effects and improving the prospects of labor. There is now extensive evidence of the efficacy and safety of this method (7–9). The obstacle to wider utilization lies in the inherent lability of prostaglandin E_2 compared to the less effective prostaglandin $F_{2\alpha}$ (10). Existing formulations in simple gels and pessaries do not provide sufficient long-term stability and involve problems concerning homogeneity, sterility, and dosage safety (11, 12).

The absence of a suitable delivery vehicle and a standardized preparation of the gel has discouraged commercial production and hindered clinical usage of the prostaglandin E_2 technique. This report presents a new prostaglandin E_2 gel delivery system and affords a definite solution to the problems afflicting the practical handling of the drug for local administration. This system consists of drug distributed in the matrix of a cross-linked starch derivative. The properties of the starch powder provide a stabilizing milieu for the labile prostaglandin E_2 and, by addition of saline, a ready-to-use gel for immediate local administration.

The safety and efficacy of the starch gel prepared with prostaglandin E_2 in cervical ripening were established in clinical trials (13), and this paper outlines the pharmaceutical aspects involved in the development of such a delivery system.

EXPERIMENTAL

Materials—Prostaglandin E_2[1](I) was used in the crystalline form. [1-^{14}C]Prostaglandin E_2[2] (specific activity 2.07 GBq/mmole) was supplied as a solution in ethanol–water (7:3). The cross-linked starch polymer[3] was supplied as a dry powder (particle size 50–100 μm). All reagents were analytical grade.

Gel Formulations—The prostaglandin E_2 gel powder was prepared under aseptic conditions. A freshly prepared, sterile-filtered solution of I (50 mg) in 80% (v/v) aqueous ethanol (25 ml) was added to 50 g of the heat-sterilized (160° for 1 hr) cross-linked starch powder. During the subsequent manual mixing for 10 min, the starch material swelled to twice its original volume. After lyophilization and homogenization, the prostaglandin E_2 gel powder was dispensed in 0.5-g portions (0.5 mg of I/dose) in sterile, disposable, 10-ml syringes.

The radiolabeled prostaglandin E_2 gel powder was prepared in a similar manner with the addition of 92.5 KBq of [1-^{14}C]prostaglandin E_2 to the solution of I. For practical reasons, this batch was prepared at one-half the scale (*i.e.*, 50 doses) and under nonaseptic conditions.

The ready-to-use-gel for clinical administration was prepared by adding 2.2 ml of sterile saline *via* a hypodermic needle to the prostaglandin E_2 gel powder in the syringe. A uniform gel consistency was obtained without agitation within 30 sec.

The prostaglandin E_2 gel fortified with X-ray contrast medium was prepared by adding 1 ml of saline and 2 ml of contrast medium (6.75 g of metrizamide[4] dissolved in 7.8 ml of 0.6 m*M* sodium bicarbonate) to

[1] The Upjohn Co.
[2] The Radiochemical Centre.
[3] Perstorp AB.
[4] Amipaque, Nyegaard & Co., Oslo, Norway.

0022-3549/80/1100-1271$01.00/0