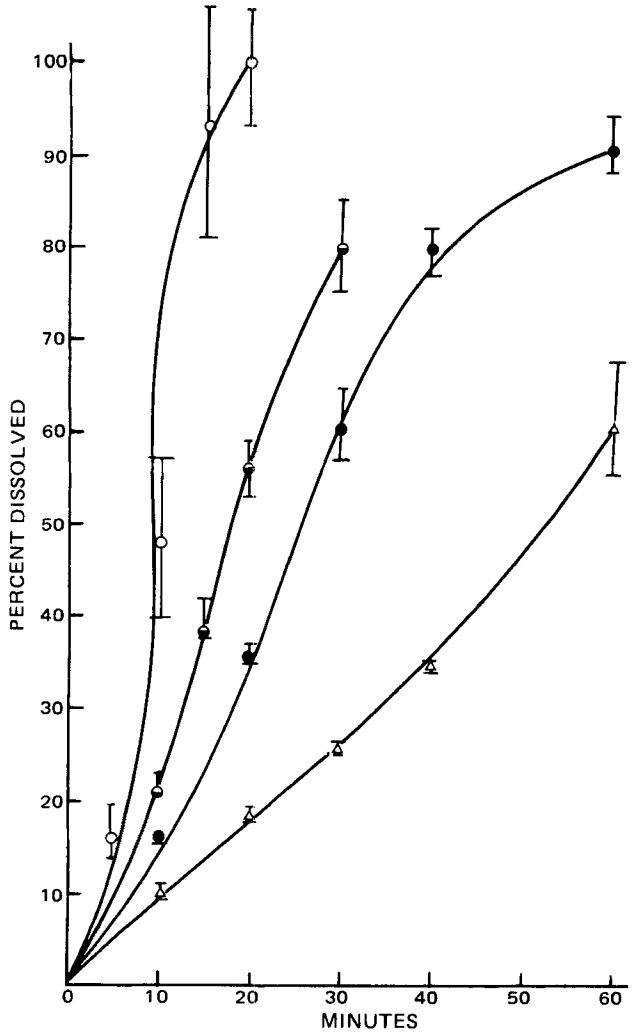

Figure 7—*Effect of shear on the drug dissolution in the procainamide hydrochloride–starch mixture (Formulation M). Key: ○, 0 min; ◒, 5 min; ●, 15 min; and △, 30 min of mixing with the intensifier bar.*

loose and packing densities of powder blends containing magnesium stearate. The powder densities increased with blending time up to maximum limiting values. These changes in the density characteristics of powders with shear are presumably related to improved flow properties and are frequently reflected in their subsequent processing, *e.g.*, in the fill weight and weight control during encapsulation.

The evidence presented in this paper illustrates the potential liabilities stemming from the indiscriminate use of shearing or mixing devices to break up aggregates in the blending operations where magnesium stearate and other hydrophobic lubricants are in the formula. The intensity of mixing is an important processing variable, capable of altering the *in vitro* availability characteristics of pharmaceutical formulations. This result, in turn, potentially may influence the bioavailability of the dosage form. A knowledge of the shear effect on drug dissolution should be an integral part of formula development and scale-up studies of drug preparations.

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High-Pressure Liquid Chromatographic Analysis of Methotrexate in Presence of Its Degradation Products

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Abstract □ Two high-pressure liquid chromatographic methods are described for the quantitative determination of methotrexate in the presence of its contaminants and degradation products. Method 1 takes less than 15 min and is recommended for routine assays of methotrexate in commercial products. Method 2 takes about 35 min and is the method of choice to detect and quantitate large amounts of degradation products. Quantitation to a level of 1 μg of methotrexate/ml is feasible by these methods and thus provides potential applicability for the analysis of methotrexate in biological fluids.

Keyphrases □ Methotrexate—high-pressure liquid chromatographic analysis, commercial preparations □ High-pressure liquid chromatography—analysis, methotrexate in commercial preparations □ Antineoplastic agents—methotrexate, high-pressure liquid chromatographic analysis in commercial preparations

Methotrexate is a folic acid antagonist widely used in cancer chemotherapy. However, methotrexate is contaminated with its degradation products and other closely related folic acid analogs. Quantitation of the contami-

nants in commercial methotrexate preparations is important because of the recent use of large doses (10–15 g) in humans.

Most methods (1, 2) for the assay of intact methotrexate in the presence of its contaminants and/or degradation products are time consuming. The USP (1) quantitative TLC method is subject to limitations of accuracy without an internal standard. Other methods such as competitive protein binding (3), although sensitive, are only suitable for biological samples because of their large coefficient of variation. A high-pressure liquid chromatographic (HPLC) method was reported for the separation of impurities in methotrexate (4). However, the quantitation of methotrexate in the presence of its degradation products was not demonstrated.

This report presents a sensitive and rapid HPLC method for the quantitative determination of methotrexate in the presence of its contaminants and degradation products.

EXPERIMENTAL

Apparatus—A liquid chromatograph[1] equipped with an injection valve[2] capable of withstanding 7000 psig was used. The valve was such that any volume less than the loop volume could be loaded directly into the loop and subsequently injected into the column without stopping the flow. The liquid chromatograph also was equipped with a fixed wavelength UV detector for monitoring the column effluent at 254 nm[3], a chart recorder[4], and an electronic integrator[5]. The columns used were 50-cm × 2.1-mm (i.d.) stainless steel tubing prepacked with anion-exchange resin[6] (30–44 μm) and 30-cm × 4-mm (i.d.) stainless steel tubing prepacked with bonded amine[7].

Reagents—USP methotrexate reference standard[8], mp 182–189°, was used.

Commercial preparations of methotrexate sodium for injection, 25 mg/ml[9], and 2.5-mg methotrexate tablets[10] also were used. Acetonitrile was a glass-distilled spectrophotometric grade[11], and all other reagents were analytical grade.

Two mobile phase solvents were used. Each liter of Solvent 1 contained 0.3 mole of sodium perchlorate, 0.15 mole of monobasic sodium phosphate, 0.15 mole of dibasic sodium phosphate, and 15 ml of acetonitrile. Each liter of Solvent 2 contained 0.075 mole of sodium perchlorate, 0.0375 mole of monobasic sodium phosphate, 0.0375 mole of dibasic sodium phosphate, and 20 ml of acetonitrile.

Unless otherwise noted, all solutions to be assayed were brought to volume with 0.1 *M* ammonia–ammonium bicarbonate buffer (pH 8.3).

Reference Solution—A reference solution of methotrexate USP was prepared by accurately weighing approximately 10 mg of methotrexate USP in a 100-ml volumetric flask and bringing to volume with 0.1 *M* ammonia–ammonium bicarbonate buffer (pH 8.3). The reference solution was freshly prepared every 2 weeks, wrapped in aluminum foil, and stored in the refrigerator (2–4°). To generate a standard curve for calculations, this reference solution was appropriately diluted.

Sample Solutions—All sample solutions were diluted to approximately 10 mg of methotrexate/100 ml and assayed.

Tablets—To minimize tablet weight variation, 10 tablets were triturated in a glass mortar and the total powder was accurately weighed. A portion of the powder equivalent to one-tenth of the total weight was then accurately weighed, placed in a 25-ml volumetric flask, and diluted to volume with 0.1 *M* ammonia–ammonium bicarbonate buffer (pH 8.3). After shaking for 10 min, the solution was filtered and assayed.

Injections—The contents of each vial were further diluted to an appropriate volume with ammonia buffer and assayed.

[1] Model 3500B, Spectra-Physics, Santa Clara, Calif.
[2] Velco type valve, Spectra-Physics, Santa Clara, Calif.
[3] Model 225, Spectra-Physics, Santa Clara, Calif.
[4] Varian A-25, Varian Associates, Palo Alto, CA 94303.
[5] Autolab System I, Spectra-Physics, Santa Clara, Calif.
[6] Vydac, E. Merck, Germany, marketed by Spectra-Physics, No. AX-107.
[7] μBondapak NH_2, marketed by Waters Associates, Milford, Mass.
[8] Lot 1260X8105, Lederle Laboratories, Pearl River, N.Y.
[9] Lot 467-164, 50 mg in 2-ml vials, Lederle Laboratories, Pearl River, N.Y.
[10] Lot 422-109, Lederle Laboratories, Pearl River, N.Y.
[11] Aldrich Chemical Co., Milwaukee, WI 53233.

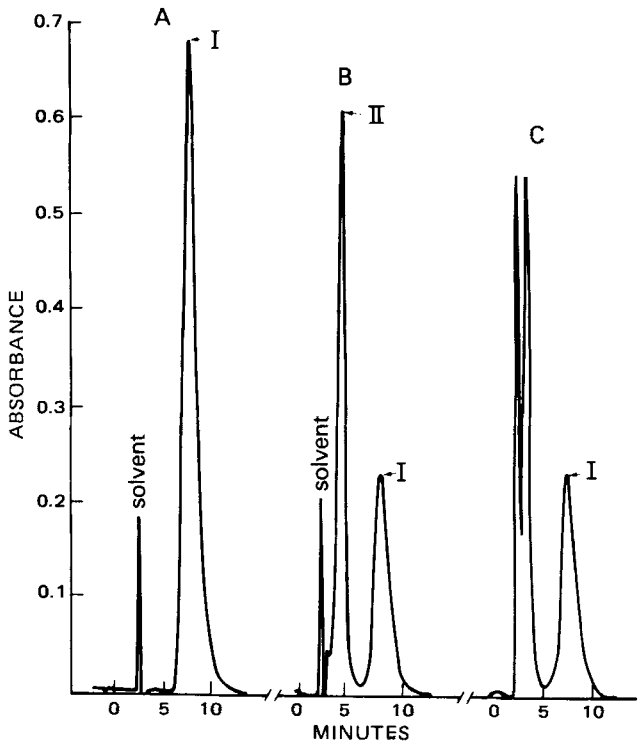

Figure 1—*High-pressure liquid chromatograms of USP methotrexate reference standard solution by Method 1. Key: A, initial; B, heat degraded, approximately 60%; C, light degraded, approximately 60%; I, methotrexate; and II,* N^{10}*-methylfolic acid.*

Heat-Degraded Solution—Approximately 10 mg of USP methotrexate reference standard was accurately weighed, placed in a 100-ml volumetric flask, and brought to volume with ammonia buffer. The solution was then packaged into ampuls, heated in an oven at 85° for 10 days, and assayed.

Light-Degraded Solution—Approximately 10 mg of USP methotrexate reference standard was accurately weighed, placed in a 100-ml flask, and brought to volume with ammonia buffer. The solution was then placed under a fluorescent light at room temperature for 6 days and assayed.

Various degradation mixtures were then made up by accurately mixing known amounts of reference solutions and heat- and light-degraded solutions.

Chromatographic Methods—*HPLC Method 1*—A 50-cm anion-exchange column[6] with the mobile phase (Solvent 1) at a flow rate of 0.6 ml/min and pressure of about 500 psi was used. A 10-μl full loop sample volume was injected quantitatively into the column with the recorder set at 0.04 full scale. After methotrexate was eluted, appearing as the last peak on the chromatogram, the column was ready for another run.

The areas obtained under the peak heights were used for the calculations. Total run time was 12–15 min. No internal standard was used, because a full loop volume was always quantitatively injected and the precision was calculated to be about 1%. Each sample run was immediately followed by a run with the reference solution.

A standard curve was prepared by determining the area ratios of different concentrations of the reference solution and plotting them against their respective known concentration ratios:

$$\text{area ratio} = \frac{\text{area of peak 1 of various dilutions of reference solution}}{\text{area of peak 1 of reference solution}} \quad \text{(Eq. 1)}$$

$$\text{concentration ratio} = \frac{\text{concentration of various dilutions of reference solution}}{\text{concentration of reference solution}} \quad \text{(Eq. 2)}$$

HPLC Method 2—Two columns, a bonded amine column[7] nearer the injection site and an anion-exchange resin column[6] in series, with the mobile phase (Solvent 2) at a flow rate of 0.8 ml/min and pressure of about 1300 psig were used. The procedure was otherwise the same as Method

Table I—Comparison of Assay Results for Intact Methotrexate in Various Samples by HPLC Method 1 and the Cellulose Column Method

Sample	Description	Methotrexate, % Expected Value	By HPLC Method 1[a]	By Cellulose Column Method
1	25.0 ml[b]	100	100	100
2	20.0 ml[b] + 5.0 ml[c]	88.4	88.34 ± 1.01	89.0
3	15.0 ml[b] + 10.0 ml[c]	76.8	76.93 ± 0.79	
4	10.0 ml[b] + 15.0 ml[c]	65.2	65.58 ± 0.94	66.7
5	5.0 ml[b] + 20.0 ml[c]	53.6	54.20 ± 0.32	
6	25.0 ml[c]	42.0	42.00 ± 0.47	42.6

[a] Average of three runs. [b] A USP methotrexate reference standard solution assigned a value of 100%. [c] A heat-degraded solution of methotrexate calculated to contain 42% methotrexate based on Sample 1 as calculated by Method 1.

1. A standard curve was prepared the same way as described for Method 1.

Cellulose Column Method—Assay on a diethylaminoethylcellulose column was performed by the method of Gallelli and Yokoyama (2), but the resin was not discarded after each run. At the end of an assay, when the last peak was eluted, the column was washed with 100 ml of 0.1 *M* ammonia–ammonium bicarbonate buffer (pH 8.3); the column was then ready for another assay. Aliquots of 10 ml of sample or reference solution were used in each run.

RESULTS AND DISCUSSION

N^{10}-Methylfolic acid is a heat-degraded product, while 2,4-diamino-6-pteridine carboxylic acid and its aldehyde are light-degraded products of methotrexate. Figure 1 shows typical chromatograms of methotrexate, light-degraded methotrexate, and heat-degraded methotrexate, using Method 1. In all three chromatograms, methotrexate was very strongly bound to the column's resin and eluted last. The light-degraded products of methotrexate eluted with the solvent front (Fig. 1C). Excellent separation between methotrexate and the major heat degradation product, N^{10}-methylfolic acid, is shown in Fig. 1B. Since the light-degraded products of methotrexate eluted with the solvent front and in no way interfered in the assay of intact methotrexate, a series of mixtures of methotrexate and heat-degraded methotrexate solutions was prepared and assayed by Method 1 and the cellulose column method. The values obtained by Method 1 were in excellent agreement with the expected values (Table I). Also, there was reasonably good agreement with the values obtained by Method 1 and the cellulose column method, although the latter values were consistently higher by about 1%.

During the analysis of various heat-degraded methotrexate solutions by Method 1, a shoulder peak (r_t = 10 min, Fig. 2*b*) appeared immediately preceding the methotrexate peak. This shoulder was especially evident when a more dilute buffer was used as the eluent (Fig. 2). Although the area of the shoulder peak was initially less than 1% of the area of the methotrexate peak, its relative percentage became increasingly important when the drug was degraded more than 70%; the shoulder peak was then 10–15% of the small methotrexate peak.

Repeated attempts using Method 1 with various buffer strengths and flow rates were unsuccessful in reproducibly separating the shoulder peak from the methotrexate peak. Success was finally achieved with a bonded amine column[7] in series with the anion-exchange resin column[6] and Solvent 2. Method 2 not only separated the shoulder peak (r_t = 13.5 min, Fig. 2*a*) from the methotrexate peak but also separated other heat degradation products more effectively, allowing for their possible quantitation.

To calculate methotrexate levels, a standard curve was prepared by plotting area ratios against concentration ratios (see *Experimental*). Although the area ratios were slightly lower than a 1:1 ratio at a concentration below 4 mg/100 ml and slightly higher than a 1:1 ratio at a concentration above 12 mg/100 ml, an excellent linear fit was obtained over a 15-fold concentration range (r = 0.9996, n = 7).

The area ratios of the various samples assayed were calculated, and their corresponding concentration ratios were determined from the standard curve. The explicit quantity of intact methotrexate in various samples was calculated as follows:

$$\text{concentration of intact methotrexate in sample} = (\text{concentration ratio})\,(R) \quad \text{(Eq. 3)}$$

$$\text{concentration of intact methotrexate in injections} = (\text{concentration ratio})\,(R)\,(2.5) \quad \text{(Eq. 4)}$$

$$\text{amount of intact methotrexate per tablet} = \frac{W_{av}}{W} \times (\text{concentration ratio})\,(R)\,(0.25) \quad \text{(Eq. 5)}$$

Table II—Results Obtained for the Assay of Methotrexate Tablets, Injectables, and Heat-Degraded and Light-Degraded Samples

Sample	Methotrexate[a], % By HPLC Method 1	By HPLC Method 2	By Cellulose Column Method
Heat degraded, approximately 60%	42.0	41.2	42.6
Heat degraded, approximately 80%	24.2	21.0	21.7
Light degraded, approximately 70%	30.4	29.4	30.2
Tablet	121.2[b] (105.1)	121.6[b] (105.4)	123.1 (106.7)
Injection	123.6[b] (107.2)	123.2[b] (107.8)	125.2 (108.5)

[a] Relative to reference solution containing 10.05 mg of methotrexate/100 ml. [b] Both tablet and injection samples contained more than 120% of the labeled amount when compared to USP methotrexate reference standard and as such violated the USP limits (90–110%). However, USP methotrexate reference standard was analyzed and found to contain 86.3% pure methotrexate (on anhydrous basis, as calculated by the method in Ref. 2). Explicit values of methotrexate present in tablets and injectables (calculated relative to USP methotrexate reference standard by the method described in this paper) should, therefore, be multiplied by 0.863 to give the true value of methotrexate content. These corrected values, represented as percent of labeled amount, are reported in parentheses.

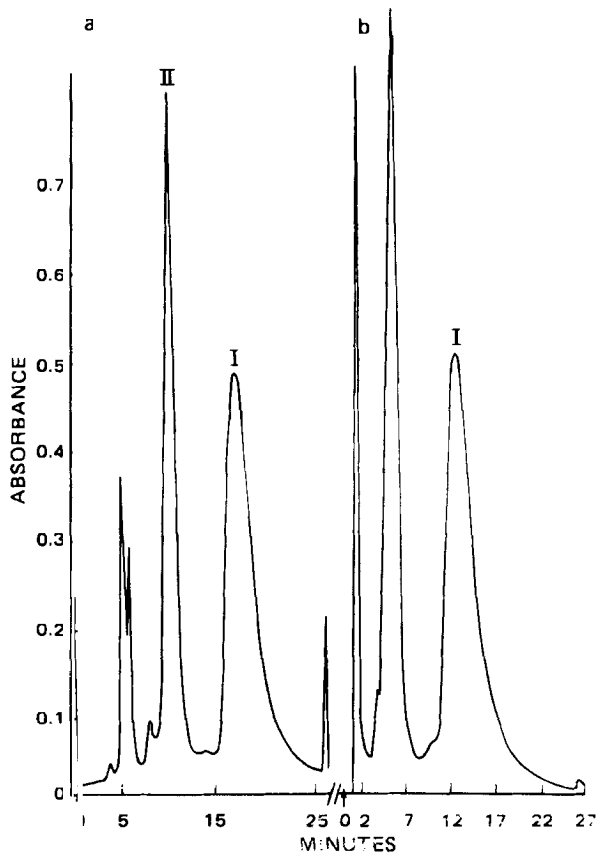

Figure 2—*High-pressure liquid chromatograms of a heat-degraded solution of methotrexate. Key:* a, *using Method 2;* b, *using Method 1 with a dilute buffer (mobile phase 2); I, methotrexate; and II,* N^{10}*-methylfolic acid.*

where R is the concentration of USP methotrexate reference solution in milligrams per 100 ml, W_{av} is the average weight of one tablet in milligrams, and W is the weight of an aliquot of the tablet triturate taken for assay.

Table II summarizes the values of various samples obtained by Methods 1 and 2 and the cellulose column method. All three methods gave essentially similar values with light-degraded methotrexate samples and less than 70% heat-degraded methotrexate samples. Therefore, considering the relative quickness of the assay procedure by Method 1 (less than 15 min), this method is recommended for routine assays of methotrexate in commercial products. Method 2 is more important in studying the kinetics of methotrexate and for quantitating its degradation products. Also, it is essential to use Method 2 in methotrexate samples that are more than 70% heat degraded.

The sensitivity of Method 1 was determined using a 100-μl sample size to detect a concentration of 0.25 μg of methotrexate/ml of solution. An aqueous solution containing 1 μg of methotrexate/ml of solution, when analyzed by Method 1, gave a recovery of 93.1 ± 4.7% of methotrexate (average of three runs). Thus, Method 1 could be potentially useful in quantitating to a level of 1 μg of methotrexate/ml in biological fluids.

The tablet excipient dye did not interfere with the assay, as seen by the close agreement between the values obtained by Methods 1 and 2 and the cellulose column method. In the latter method, the dye could be seen as a yellow band remaining at the top of the cellulose column. The benzyl alcohol preservative present in the injectables is nonionic and eluted almost with the solvent front. Other preservatives such as methylparaben and propylparaben eluted immediately following the solvent front and did not interfere with the assay of intact methotrexate.

In all of the described assays, the amount of intact methotrexate in the unknown is calculated as a percentage of the reference solution. The explicit quantities are then calculated from the known concentration of methotrexate USP in the reference solution. However, methotrexate USP has been reported (2) to be only 85–90% pure. Therefore, explicit quantities in the assays, which are calculated by assuming methotrexate USP as 100% pure, must be interpreted accordingly. However, in the determination of relative amounts in various preparations or in the determination of percentage degradation, this consideration is obviated.

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Vehicle Effects on Ocular Drug Bioavailability II: Evaluation of Pilocarpine

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Abstract □ The influence of vehicle composition on ocular penetration of pilocarpine was studied in the albino rabbit. Increasing the pH of a vehicle promoted increased corneal penetration for pilocarpine, in accordance with the pH–partition hypothesis, but a similar series of experiments with a nonionizable drug, glycerin, gave similar results. The extent of pH-induced lacrimation by the vehicle and its effect on precorneal drug concentration also was determined. Increased pilocarpine absorption at neutral to slightly alkaline pH was due primarily to its peculiar solubility characteristics coupled with less irritation and lacrimation rather than a direct pH effect on the molecule. Incorporation of pilocarpine into a petrolatum-based ointment vehicle resulted in increased aqueous humor pilocarpine levels above those provided by an equivalent dose of aqueous solution. The mechanism of this increase was determined to be a higher effective concentration of pilocarpine in the ointment vehicle coupled with an increase in contact time of the dose. The ointment system also exerted an unusual form of vehicle control in that it promoted the corneal penetration of pilocarpine while impeding uniform mixing of the dose with tears and thereby imposed a restriction on the amount of pilocarpine available to the ocular tissues.

Keyphrases □ Pilocarpine—effect of vehicle composition and pH on ocular bioavailability, rabbits □ Vehicles—effect of composition and pH on ocular bioavailability of pilocarpine, rabbits □ Bioavailability, ocular—pilocarpine, effect of vehicle composition and pH, rabbits □ Ocular bioavailability—pilocarpine, effect of vehicle composition and pH, rabbits □ Ophthalmic cholinergic agents—pilocarpine, effect of vehicle composition and pH on ocular bioavailability, rabbits

The first report in this series dealt with vehicle influence on the ocular bioavailability of the relatively water-insoluble steroid fluorometholone (1). In that study, mechanisms of vehicle effects on ocular bioavailability were based on the interplay of the steroid and vehicle properties with the physiological and physicochemical nature of the drug administration site, *i.e.*, the precorneal portion of the eye. The present study extended this work to include the important water-soluble antiglaucoma drug pilocarpine. Thus, this examination of a water-soluble drug, along with earlier work on the relatively water-insoluble steroid, provides considerable perspective on factors that should be considered when formulating an ophthalmic drug for topical delivery to the eye to obtain maximum drug benefit.

BACKGROUND

During the past 30 years, numerous vehicles have been screened for various drugs to improve the overall intraocular penetration of topically applied drugs. Because of an inadequate understanding of the interplay between drug–vehicle–precorneal area properties, the primary thrust of much research has been directed toward prolonging the presence of the instilled dose in the precorneal area, usually referred to as increasing the contact time. Most often, these formulations rely mainly upon the viscosity character of the vehicle and its subsequent rheological effect on drainage loss of the dose *via* the nasolacrimal duct. Thus, most topically applied ocular vehicles have included viscous polymers such as hydroxypropylcellulose (2–4), methylcellulose (5–12), and polyvinyl alcohol (2–4, 6–8, 13–15).

Detailed studies of the drainage loss rate of these vehicles also have been performed (3, 5, 13, 16, 17) to permit selection of vehicles exhibiting