Sensitive High-Performance Liquid Chromatographic Method for the Determination of Etodolac in Serum

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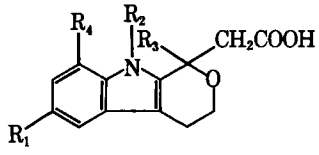
Abstract □ A sensitive high-performance liquid chromatographic method for the determination of etodolac in serum was developed. The limit of detection was 0.2 μg/ml. The specificity of the method was demonstrated by the lack of response obtained with a variety of control sera, sera spiked with etodolac congeners, and sera obtained from rats treated with a variety of other drugs.

Keyphrases □ Etodolac—sensitive high-performance liquid chromatographic method for determination in serum □ High-performance liquid chromatography—sensitive method for the determination of etodolac in serum □ Serum specificity—sensitive high-performance liquid chromatographic method for the determination of etodolac □ Analgesics—etodolac, sensitive high-performance liquid chromatographic method for determination in serum □ Anti-inflammatory agents—etodolac, sensitive high-performance liquid chromatographic method for determination in serum

Experiments in laboratory animals have demonstrated that etodolac [1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indole-1-acetic acid[1], (I)] is a potent nonsteroidal, anti-inflammatory and analgesic agent (1, 2). To carry out pharmacokinetic studies in both animals and humans, a



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sensitive and reliable analytical method was needed. Etodolac can be quantitated by a fluorometric method that has been developed for its 1-propyl congener, prodolic acid (3); however, because of endogenous fluorescent substances, the limit of detection is only 2–3 μg/ml. Therefore, a specific and sensitive (0.2 μg/ml) high-performance liquid chromatographic (HPLC) method has been developed.

EXPERIMENTAL

Reagents—Isopentyl alcohol[2], hexane[3], methanol[3], and acetonitrile[4] were used as supplied. All other chemicals, monobasic potassium phosphate, hydrochloric acid, phosphoric acid, and glycine, were analytical reagent grade. Etodolac and its 1-propyl, 1-phenyl, 1,9-dimethyl, and 6-hydroxy congeners were synthesized as described previously (4, 5) as was [^{14}C]etodolac (6). Drugs used for specificity studies were purchased from commercial sources.

Instrumentation—The liquid chromatographic system employed consisted of an automatic variable-volume injector[5], a high-pressure pump[6], a variable wavelength spectrophotometer[7], and a recorder[8].

Column Preparation—Two 250 × 4.6-mm stainless steel HPLC columns were employed in the experiments: Chromosorb LC-7[9] (ODS, 10 μm; column A) and Spherisorb[10] (ODS, 5 μm; column B). Using 2-propanol slurries, the columns were packed at 6000 psi with a compressed air pump[11]. The columns were mounted with a glass water-jacket to provide temperature control. At a flow rate of 1.8 ml/min and a temperature of 50°, typical back pressures of 750 and 1680 psi were obtained for columns A and B, respectively.

Extraction and Mobile Phases—For both columns, the mobile phase was a mixture of 0.1 *M* potassium phosphate (pH 6.0) and acetonitrile. To provide similar retention times, the concentration of acetonitrile was varied from 30% (column B) to 38% (column A). The extraction phase was prepared by mixing isopentyl alcohol and hexane in a ratio of 1:19 (v/v).

Standards—A stock standard of 1.0 mg/ml of etodolac was prepared by dissolving 10.0 mg in 1.0 ml of methanol and completing to 10.0 ml with distilled water. Dilute, nonextracted test-tube standards were prepared at the desired concentration by appropriate dilution in 0.1 *M* glycine buffer (pH 11.0). In recovery studies and in actual analytical runs, working standards were prepared by diluting the stock standard in pooled rat, dog, or human control serum.

Procedure—Solutions of 4.0 ml of 1.0 *N* HCl and 5.0 ml of isopentyl alcohol–hexane were added to 1.0 ml of serum (or plasma) in a 15-ml screw-capped glass[12] tube. The tube was capped and agitated mechanically for 15 min. The two phases were separated by low-speed centrifugation (5 min, 1000 rpm), and 4.0 ml of the upper phase was transferred to a clean tube, and 1.0 ml of 0.1 *M* glycine buffer[13] was added. The tube was agitated for an additional 15 min, and a 0.8-ml aliquot of the aqueous phase was transferred to a clean tube. Prior to injection onto the HPLC column, 20 μl of 2.5 *M* phosphoric acid was added to partially neutralize the glycine buffer. With each analytical run, column performance was monitored by injecting test-tube standards. Control serum blanks and control serum, spiked with the appropriate concentration of etodolac (in triplicate), were carried through the method.

Chromatographic conditions were as follows: injected sample volume, 50–150 μl; temperature, 50°; flow rate, 1.8 ml/min; mobile phase, acetonitrile–phosphate buffer (30 or 38% for columnns B or A, respectively); UV detector settings, 226 nm and 0.05 AUFS (running at high sensitivity). Quantitation was based on peak height of the unknowns relative to that of the spiked control serum standards.

Recovery—Human serum spiked with 1.0–10.0 and 5.0–50.0 μg/ml of etodolac was carried through the procedure, in triplicate, to provide data on the linearity and reproducibility. Recovery was based on the response of test-tube standards injected concurrently. Recovery from a variety of aqueous media, *i.e.*, rat, dog, or human serum, and distilled water was also carried out on replicate samples at concentrations of 1.0, 10.0, and 50.0 μg/ml.

Sensitivity and Specificity—The specificity of the procedure was evaluated using three criteria: (*a*) interference due to similar chromatographic characteristics (retention times) and spectral properties (UV absorption at 226 nm) of endogenous constituents of serum, (*b*) the potential for interference from etodolac metabolites (or congeners), and (*c*) interference from other drugs that may be administered concurrently.

Typical serum blanks were measured by processing replicate (10–23) samples of rat, dog, and human control sera through the method.

Interference from etodolac congeners was estimated by injecting the pure compounds and evaluating their retention times relative to that of etodolac.

[1] Ultradol, Ayerst Laboratories, Inc., New York, N.Y.
[2] Fluorometric grade, A & C American Chemicals.
[3] Spectrograde, A & C American Chemicals.
[4] Lichrosolv, BDH Chemicals.
[5] Waters Associates, WISP model 710.
[6] Altex Scientific, Inc., model 110A.
[7] Perkin-Elmer, model LC-55B.
[8] Perkin-Elmer, model 56.
[9] Johns-Manville.
[10] Phase Separation Ltd.
[11] Haskel Eng. and Supply Co., model DST-1220.
[12] Pyrex tube.
[13] Due to complex formation with an impurity in some grades of glycine, etodolac may chromatograph as a split peak. The glycine buffer can be replaced with 0.1 *M* THAM [tris(hydroxymethyl)amino methane] (Fisher Scientific Co.) (pH 11.0).

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Table I—Recovery of Etodolac [a] from Water and Rat, Dog, and Human Sera

Medium	n	Mean ± SE
Water	18	93.1 ± 1.0
Sera		
Rat	17	86.2 ± 1.7
Dog	18	88.0 ± 1.2
Human	18	91.5 ± 2.1

[a] Six replicates of 1.0, 10.0, and 50.0 μg/ml.

In random serum samples from rats and dogs given etodolac orally at a 50-mg/kg dose, etodolac concentrations were estimated by the fluorometric procedure (3) and in a second aliquot by the HPLC procedure (using a 5 μm Lichrosorb RP-2 column).

In selected studies in rats and dogs (7), [^{14}C]etodolac was administered orally in doses of 10 and 50 mg/kg, and total carbon 14 and unchanged etodolac were estimated at 0.25, 0.5, 1, and 3 hr postdosing.

Interference from a concurrently administered drug, or its metabolite(s), was estimated by treating rats with a high dose of the drug, obtaining a blood sample 2 hr postdosing, and processing the serum through the etodolac method. Interference was estimated from the response, at the retention time of etodolac, compared to that of serum from saline-treated control rats. Drugs tested and dose administered, in milligrams per kilogram, were: ethacrynic acid (300), salicylic acid (400), acetominophen (400), niacin (300), propoxyphene (60), phenobarbital (100), hydrochlorothiazide (100), tolbutamide (200), glyburide (200), indomethacin (25), phenylbutazone (200), phenytoin (100), dicumarol (250), and diazepam (350).

Stability—The stability of etodolac in serum was determined by analyzing serum samples spiked at 60.0 μg/ml after 1-, 3-, and 7-day storage at 20° or in the refrigerator at 4°.

RESULTS AND DISCUSSION

Chromatography—Typical liquid chromatograms of an etodolac standard, a control serum extract, and an extract of control serum spiked with etodolac are presented in Fig. 1. Using column B (250 × 4.6-mm) and a mobile phase of 30% acetonitrile–phosphate buffer, etodolac had a retention time of 5.0 min (flow rate, 1.8 ml/min). No interfering peaks were seen in the scan obtained with control serum. With column A a similar retention time is obtained if the concentration of acetonitrile is increased to 38%.

The linearity in the peak height response of the detector was demonstrated for low (1–10 μg/ml) and high (5–50 μg/ml) concentrations of etodolac. The regression line for the low test-tube standards was: $y = 21.4x - 1.23$ ($R = 0.9999$). For the high concentration standards the regression line was: $y = 4.79x - 3.93$ ($R = 0.9996$).

Recovery Studies—The linearity of recovery of etodolac from pooled human serum was also tested at two concentration ranges. For triplicate samples, covering the range of 1.0–10.0 μg/ml, the recovery was independent of etodolac concentration. The regression line for the recovery was: $y = 10.28x - 0.39$ ($R = 0.9984$). At high concentrations (5–50 μg/ml), linearity was maintained [regression line: $y = 2.57x - 3.55$ ($R = 0.9970$)]. In separate experiments small differences were noted when recoveries from rat, dog, and human sera and distilled water were evaluated at concentrations of 1.0, 10.0, and 50.0 μg/ml (Table I). The highest recovery (93.1%) was obtained with water, and with the sera recoveries varied from 86.2 (rat) to 91.5% (humans). Over a period of 6 months to 1 year, recovery of etodolac from pooled human serum varied from a low of 70 to a high of 94% [mean ± SE, 81.0 ± 1.6%]. The variations in day-to-day recovery from the same pooled serum were of the same order of magnitude as the differences found for various sera.

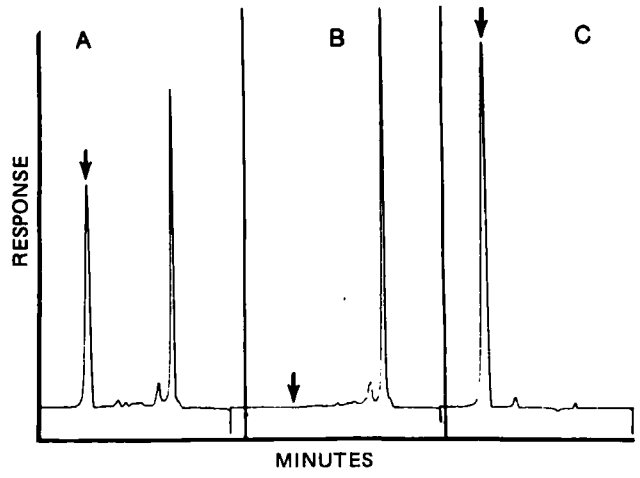

Figure 1—*HPLC chromatograms of: (A) extract of control serum spiked with etodolac (5 μg/ml); (B) extract of human control serum; and (C) etodolac standard (5 μg/ml), retention time (↓) 5.0 min, column 5 μm Spherisorb ODS.*

In the course of these experiments, it became apparent that variations in recovery were to a large extent due to instability of the etodolac test-tube standards (see *Stability*). To ensure that the small day-to-day and species-to-species variations in recovery do not result in an error in the estimation of etodolac, in actual analytical runs, standardization is based on appropriate serum standards spiked and run concurrently.

Sensitivity and Specificity—Control sera from rats ($n = 10$), dogs ($n = 23$), and humans ($n = 17$) were processed through the method and injected on the liquid chromatograph, operating at its highest sensitivity (0.05 AUFS). With the exception of one spurious sample (the apparent etodolac concentration was 0.7 μg/ml) none of the extracts revealed any UV absorption in the region of emergence of etodolac. The limit of detection of the method, consistent with baseline noise, was conservatively taken as 0.2 μg/ml. In contrast to the fluorometric procedure (3) with respect to endogenous substances, the HPLC method is specific.

When randomly selected serum samples were analyzed by both the fluorometric (3) and HPLC procedures, a good correlation was found. The mean etodolac concentrations in dog serum were estimated as 20.8 (fluorometric) and 18.3 μg/ml (HPLC). For rats the corresponding values were 78.7 and 80.2 μg/ml. The differences of 2.2 ± 1.2 and −1.5 ± 1.1 μg/ml were within the limit of reproducibility of the fluorometric method. When higher concentrations of etodolac are being measured, the more rapid fluorometric procedure can be employed, as long as the values are corrected for the 2–3 μg/ml of apparent etodolac found in control sera.

When sera from rats and dogs treated with [^{14}C]etodolac were analyzed for unchanged drug and total carbon 14 (Table II), 85–95% of the carbon 14 was accounted for by unchanged etodolac. In serum extracts used for chemical analysis, the percentage would be even higher. This explains why the inherently more specific HPLC method and the fluorometric method provide similar values. Finally, the ready separation of etodolac from its congeners, especially the phenolic analogue (Table III), suggests that the method is specific with regard to potential metabolites.

The potential for interference from selected drugs or their metabolites with the HPLC method was tested as follows. Rats were given high doses of each drug, bled 2 hr postdosing, and the serum was processed through the etodolac procedure. The HPLC chromatograms were examined for the presence of interfering peaks. Serum from control rats served as reference. The sample extracts were injected on both columns A and B. When ethacrynic acid, niacin, propoxyphene, phenobarbital, hydro-

Table II—Serum Concentrations of Unchanged Etodolac [a] and Total Carbon 14 in Rats and Dogs Given [^{14}C]Etodolac Orally

	Rat [b]			Dog [c]		
Postdose, hr	Etodolac, μg/ml	Carbon 14, μg/ml	Ratio	Etodolac, μg/ml	Carbon 14, μg/ml	Ratio
0.25	17.2	18.0	0.96	64.0	71.0	0.90
0.50	23.8	24.6	0.97	111.3	121.5	0.92
1.00	30.0	30.9	0.97	141.3	157.4	0.90
3.00	23.0	25.7	0.89	41.3	53.5	0.75
Mean			0.95			0.87

[a] *Fluorimetric* analysis. [b] $n = 4$, dose = 10 mg/kg po. [c] $n = 4$, dose = 50 mg/kg po.

Table III—Relative Retention Times [a] of Etodolac Congeners

Compound	Substituents R_1	R_2	R_3	R_4	Relative Retention Time
Etodolac	H	H	C_2H_5	C_2H_5	1.00
I	H	H	n-C_3H_7	H	0.80
II	H	H	phenyl	H	0.94
III	H	CH_3	CH_3	H	0.62
IV	OH	H	CH_3	H	0.44

[a] Chromosorb column; mobile phase: 38% acetonitrile–phosphate buffer; etodolac retention time, 5.0 min.

Table IV—Stability of Etodolac in Serum [a]

Temperature	Recovery (%) on day 1	3	7
20°	100.5 ± 0.5	97.6 ± 0.4	100.2 ± 0.5
4°	98.6 ± 0.5	97.4 ± 0.5	100.4 ± 0.6

[a] Pooled serum spiked with 60 μg/ml of etodolac.

chlorothiazide, glyburide, or diazepam were administered, no peaks were seen in the HPLC scans. Phenylbutazone and dicumarol produced strong interfering peaks with either column. With indomethacin, acetominophen, and salicylate, peaks separate from etodolac were noted, but due to tailing, these peaks partially overlapped the etodolac peak when injected on column A. A better separation was achieved on column B. Because concomitant administration of salicylate or acetominophen is likely to occur clinically, pooled rat serum spiked with etodolac was supplemented with 50–500 μg/ml of salicylate or 20–250 μg/ml of acetominophen, carried through the HPLC procedure, and analyzed on column B. No interference could be demonstrated, nor was there any adverse effect on the recovery of etodolac. In the presence of coadministered drugs, column B is preferred.

Stability—When etodolac was added to pooled serum and kept for up to 7 days at room temperature (20°) or in the refrigerator (4°), no significant loss of etodolac was detected at either temperature (Table IV).

Because of day-to-day variations in recovery, the stability of the test-tube standards and the extracted spiked control serum standards was also investigated. The peak height response of the test-tube standards (n = 12) declined from 100.0 ± 1.3 to 91.5 ± 1.2% in 1 hr. In contrast, the extracts from spiked control serum remained unchanged: 100.0 ± 1.3 *versus* 100.7 ± 2.2%. For this reason, the test-tube standard is used only to adjust mobile phase concentrations when the method is being set up. Quantitation is based on the stable extracts from spiked control sera, and no recovery factor is needed.

Application of the method—Initial studies in humans have indicated that activity is achieved with 100-mg etodolac doses given twice daily. At these doses the peak concentrations of etodolac varied between 4.0 and 14.3 μg/ml, and the daily minima between 0.3 and 3.5 μg/ml. Thus, the HPLC procedure possesses the specificity and sensitivity required to monitor etodolac concentrations in humans.

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Analysis of Chlorobutanol in Ophthalmic Ointments and Aqueous Solutions by Reverse-Phase High-Performance Liquid Chromatography

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Abstract □ A reverse-phase high-performance liquid chromatographic assay for chlorobutanol was developed and found suitable for the routine analysis of ophthalmic ointments and aqueous solutions. The method utilized a column packed with 10-μm octadecylsilane with a mobile phase of methanol–water (50:50). Peak detection was by UV absorption at 210 nm. In this chromatographic system, chlorobutanol had a capacity factor (K') of 4.1. Standard curves obtained in the presence of ointment vehicle containing an aminoglycoside were linear, intercepted at zero, and averaged 99.4% recovery. Degradation studies indicated that the method was stability indicating. The analytical results for a complete experimental ophthalmic ointment and an aqueous ophthalmic diluent are presented. This high-performance liquid chromatographic method of analysis represents an alternative to GC procedures for determining chlorobutanol.

Keyphrases □ Chlorobutanol—analysis in opthalmic ointments and aqueous solutions by reverse-phase high-performance liquid chromatography □ High-performance liquid chromatography—reverse-phase, analysis of chlorobutanol in ophthalmic ointments and aqueous solutions □ Ophthalmic ointments—aqueous solutions, chlorobutanol, analysis by reverse-phase high-performance liquid chromatography □ GC—alternative method for analysis of chlorobutanol in opthalmic ointments and aqueous solutions, reverse-phase high-performance liquid chromatography

Chlorobutanol (1,1,1-trichloro-2-methyl-2-propanol) is a commonly used preservative in ophthalmic medications. It is readily degraded by base as shown in Scheme I to form acetone, carbon monoxide, and chloride ion (1). Chlorobutanol is also highly volatile, and loss by evaporation from ophthalmic solutions was reported through porous plastic bottles and closures (2).

BACKGROUND

A variety of analytical methods were developed for the analysis of chlorobutanol. Originally, analysis consisted of decomposing chlorobutanol by heating with base and then analyzing for chloride ion. This was done titrametrically (3, 4) iodometrically (5), gravimetrically (6), and amperometrically (7). Degradation using a known amount of base and determination of excess base by acid titration and degradation followed by an iodometric determination of acetone (8) also were used. These

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