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ISOMODAL COLUMN SWITCHING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC TECHNIQUE FOR THE ANALYSIS OF CIGLITAZONE AND ITS METABOLITES IN HUMAN SERUM

J.W. COX* and R.H. PULLEN

Drug Metabolism Research, Pharmaceutical Research and Development, The Upjohn Company, 301 Henrietta Street, Kalamazoo, MI 49001 (U.S.A.)

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SUMMARY

The application of isomodal column switching high-performance liquid chromatography as an alternative to gradient elution was investigated for the analysis of ciglitazone, a potential oral antidiabetic agent, and its monohydroxyl metabolites in human serum. A high-performance liquid chromatographic apparatus was designed to perform on-line fractionation of the serum extract into non-polar (drug) and polar (metabolite) fractions which were then automatically routed into individually optimized, isocratic, reversed-phase high-performance liquid chromatographic systems for simultaneous analysis. Sample fractionation was performed with a reversed-phase guard column, and solvent routing was accomplished with microprocessor-controlled switching valves. Serum was extracted for analysis by a one-step mode sequencing procedure using disposable bonded-phase columns, and quantitation was accomplished with spiked serum standards. Performance specifications of the method were defined for precision, accuracy, linearity, and sensitivity. The column switching method was found to be both expedient and reliable, and it may have general utility for the routine, quantitative analysis of drug/metabolite mixtures that cannot be assayed by simple isocratic elution methods.

INTRODUCTION

Ciglitazone is a potential oral antidiabetic agent intended for non-insulin-dependent diabetics [1, 2]. Both the drug and its known hydroxyl/oxo metabolites have shown pharmacological activity in insulin-resistant animal models, and it is therefore of interest to monitor serum levels of both ciglitazone and its metabolites in human clinical trials. As described below, resolution of the individual monohydroxyl metabolites by high-performance liquid chromatography (HPLC) was achievable only with a reversed-phase system, on which

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there was a large difference between the capacity factors of the parent drug and the metabolites.

This is an example of the general elution problem described by Snyder [3], i.e., the problem of analyzing a sample for multiple components that have widely spaced capacity factors. There are two general methods of dealing with this problem when analyzing multicomponent samples by HPLC. In the first, the sample or sample extract is divided into separate portions for individual isocratic analysis of the more poorly retained and strongly retained components. Late eluting peaks that otherwise might interfere with the poorly retained component assay are eliminated by either pre-analysis cleanup (e.g., by thin-layer chromatography) or column backflushing techniques [4, 5]. Because of the increased labor, sample, and equipment requirements imposed by making two sample injections, this approach is often unsuitable for routine, quantitative analysis.

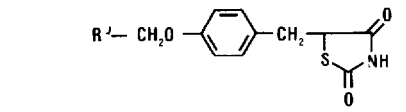
The second method of dealing with the general elution problem is to use techniques that require only a single sample injection. These include gradient elution procedures (programming the mobile-phase composition, flow-rate, or column temperature) [6], and column switching techniques [7—9]. The latter category includes isomodal sequencing methods, such as stationary-phase programming, that adjust component capacity factors by linking columns of the same type but with different surface area or loading [10] or length [11]. These methods are called isomodal because they employ a single chromatographic mode, or physical basis for separation. Heteromodal sequencing techniques, which greatly increase the peak capacity and selectivity of a system by linking different chromatographic modes [12, 13], have limited application to the general elution problem. The most cost-effective means of solving the general elution problem will depend on the application; for routine, quantitative assays, isomodal column switching methods were judged to be superior to other methods in terms of reliability, cost, and speed [6].

The general elution problem is frequently encountered in drug metabolism studies, where there can be large polarity differences between a drug and its metabolites. Nevertheless, the primary application of HPLC column switching in the pharmaceutical area has been intended to reduce analysis time when monitoring for the parent drug only and not to assist in multicomponent drug/metabolite assays [4, 9, 13—16]. The aim of the present study was to test the applicability of column switching techniques for the routine, quantitative analysis of ciglitazone and its monohydroxyl metabolites in human serum. The method employs a one-step serum extraction procedure and an automated, isomodal, column switching HPLC system designed for the simultaneous isocratic elution of the drug and its metabolites. The performance and dependability of the method are evaluated and its utility is demonstrated in a clinical trial.

EXPERIMENTAL

Materials

Ciglitazone {Fig. 1; 5-[4-(1-methylcyclohexylmethoxy)benzyl] thiazolidine-2,4-dione; U-63,287}, *cis*-4′-ol {5-[4-(*c*-4-hydroxy-1-methyl-*r*-1-cyclohexyl-



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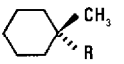
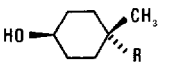
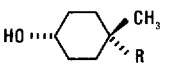
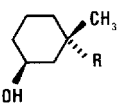
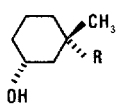
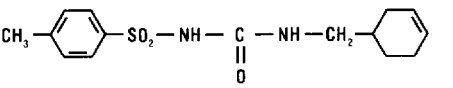
Ciglitazone	
trans-4′-ol	
cis-4′-ol	
trans-3′-ol	
cis-3′-ol	

Fig. 1. Structures of ciglitazone and its monohydroxyl metabolites.

Internal Standards

U-11,824



U-53,059

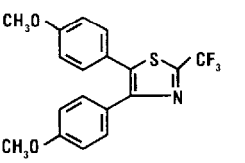


Fig. 2. Structures of the internal standards used for the metabolite assay (U-11,824) and the ciglitazone assay (U-53,059).

methoxy)benzyl] thiazolidine-2,4-dione}, *trans*-4′-ol {5-[4-(*t*-4-hydroxy-1-methyl-*r*-1-cyclohexylmethoxy)benzyl] thiazolidine-2,4-dione}, *cis*-3′-ol {5-[4-(*c*-3-hydroxy-1-methyl-*r*-1-cyclohexylmethoxy)benzyl] thiazolidine-2,4-dione}, and *trans*-3′-ol {5-[4-(*t*-3-hydroxy-1-methyl-*r*-1-cyclohexylmethoxy)benzyl]-thiazolidine-2,4-dione} were provided by Takeda Chemical Industries (Osaka, Japan). The internal standard for ciglitazone analysis, U-53,059 [Fig. 2; 4,5-bis(*p*-methoxylphenyl)-2-(trifluoromethyl)thiazole] and the internal standard for the metabolite analysis, U-11,824 [Fig. 2; 1-(3-cyclohexen-1-ylmethyl)-3-*p*-tolylsulfonylurea] were supplied by the Pharmaceutical Research and Development Labs. of Upjohn (Kalamazoo, MI, U.S.A.). Acetonitrile, hexane, methanol and water were UV or HPLC grade and were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Bond Elut™ C_{18} (100 mg/1.0 ml) and silica (500 mg/2.8 ml) extraction columns were purchased from Analytichem International (Harbor City, CA, U.S.A.).

Instrumentation

The HPLC apparatus consisted of two isocratic systems linked via a common guard column (Fig. 3). Injections were made automatically with an Upjohn μP autosampler (Upjohn; not commercially available). Pumps A and B (Beckman 112 solvent delivery modules, Beckman Instruments, Berkeley, CA, U.S.A.) were connected to Valco 10-port and 6-port air-actuated (4 bar)

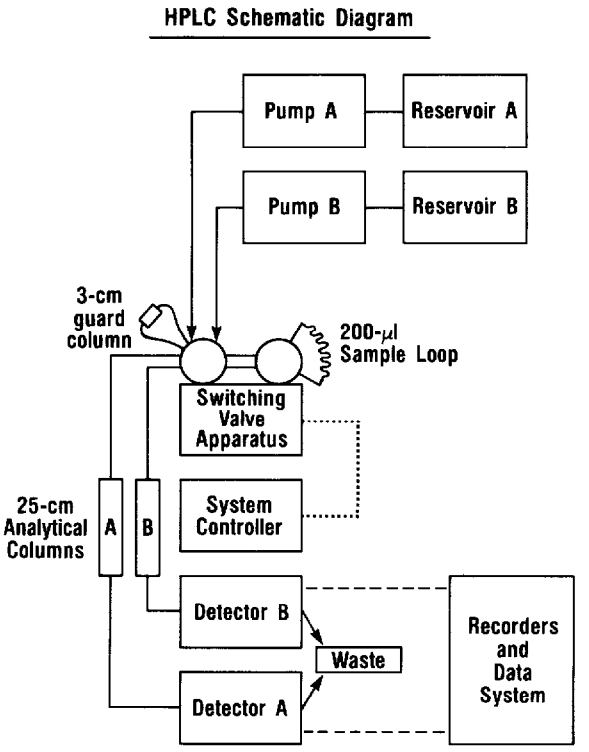


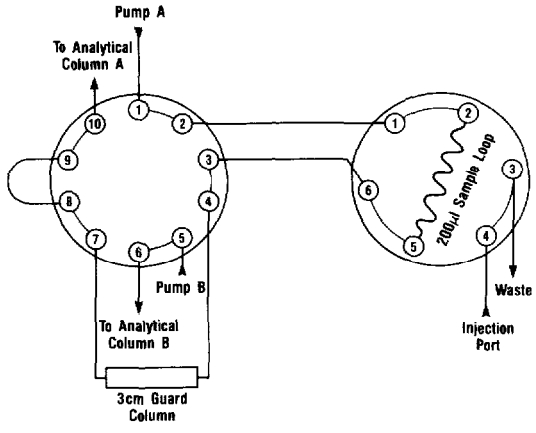
Fig. 3. Schematic representation of the HPLC apparatus.

switching valves (Valco Instruments, Houston, TX, U.S.A.). The position of these valves was controlled by a Beckman 421 system controller, which has built-in external flags for the control of external devices. The system controller was interfaced to the switching valves by a solenoid valve/solid state relay device assembled by the Research Technical Services Unit, Pharmaceutical Research and Development, Upjohn. Three switching valve positions were employed for this assay (Fig. 4): position III permitted loading of the sample loop and reconditioning of the guard column; position I injects the sample onto the guard column, with the guard column on-line in HPLC system A; and position II switches the guard column in-line with HPLC system B, and is used to inject guard column retentate from the initial injection into HPLC system B. The guard column was a Brownlee RP-18 Spheri-5, 3-cm cartridge (Brownlee Labs., Santa Clara, CA, U.S.A.). Analytical columns A and B were Supelcosil® LC-18, 5 µm, 250 × 4.6 mm I.D. (Supelco, Bellefonte, PA, U.S.A.). Detector A was an LDC UV Monitor III, Model 1203A, equipped with a Cd lamp (229 nm) (Laboratory Data Control, Riviera Beach, FL, U.S.A.). Detector B was a Beckman 165 variable-wavelength detector (229 nm).

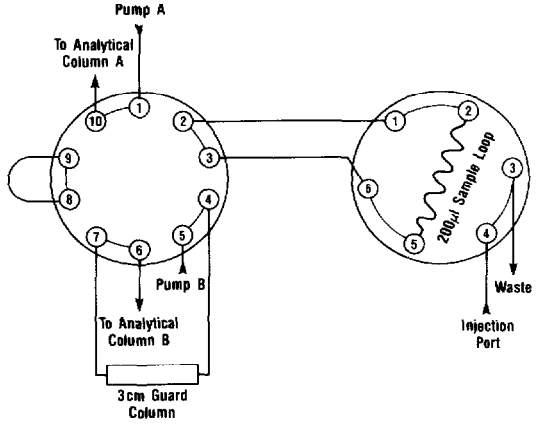
Peak height and area measurements, response factor calculations, linear regression analysis, and unknown quantitation calculations for both detectors were performed using the Upjohn chromatography system on a Harris 500 computer.

Switching Valve Configurations

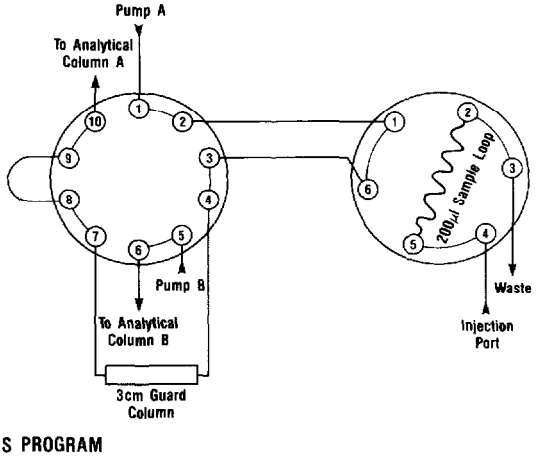
POSITION I
Inject the sample with guard column in-line with system A.



POSITION II
Inject the guard column retentate into system B.



POSITION III
Re-equilibrate guard column and load the sample loop.



ANALYSIS PROGRAM
Position I: 0-5 min. (External Flag 1 at 0 min.)
Position II: 5-20 min. (External Flag 2 at 5 min.)
Position III: 20-25 min. (External Flags 3 and 4 at 20 min.)

Fig. 4. Schematic representation of the switching valve configurations used to complete one analysis cycle.

HPLC conditions

Mobile phase A (Fig. 3) was acetonitrile—7 m*M* phosphoric acid (aq.), pH 2.5 (4:6). Mobile phase B was acetonitrile—7 m*M* phosphoric acid (aq.), pH 2.5 (7:3). Phosphate buffer (pH 2.5) for the mobile phases was prepared by diluting 0.50 ml of 85% phosphoric acid to 1.0 l with water. The water (HPLC grade, Burdick and Jackson Labs.) used to prepare mobile phase A was extracted twice with a 0.1-volume of hexane in a separatory funnel in order to remove non-polar contaminants which otherwise accumulated on the guard column and interfered with the system B analysis. For the same reason, mobile phase A was not filtered through an ultrafiltration system prior to HPLC. Membrane filters from several suppliers were found to contaminate the mobile phase with non-polar components. Mobile phase B was filtered through 0.45-μm Nylon 66 membrane filters (Rainin Instrument, Woburn, MA, U.S.A.). Both mobile phases were degassed by helium purging. Mobile-phase flow-rates were 1.4 ml/min and 1.25 ml/min for mobile phases A and B, respectively, and both pumps operated continuously throughout the analysis cycle. The guard and analytical columns were at ambient temperature. The injection volume was 0.2 ml.

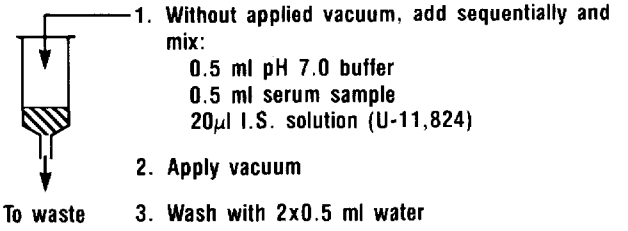
Serum extraction

Blood was collected from human volunteers by venipuncture and stored at 4°C for 3 h. Serum was collected by centrifugation in a refrigerated centrifuge for 15 min at 1300 *g*. Serum samples were transferred immediately to 15-ml screw-capped vials fitted with aluminium-lined caps and frozen at −20°C.

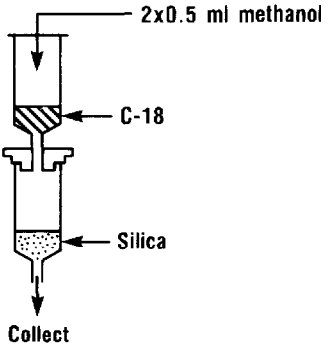
Bond Elut columns were prepared for serum extraction by the following procedure: C_{18} columns were washed with 2 ml of methanol followed by 2 ml of hexane-washed water (see HPLC conditions); silica gel columns were washed with 5 ml of hexane followed by 5 ml of methanol. The washed C_{18} columns were connected to the vacuum manifold (J.T. Baker, Phillipsburg, NJ, U.S.A.) and, without applying vacuum, 0.5 ml of 1.0 *M* phosphate buffer (pH 7.0) was added to each followed by 0.5 ml of the thawed serum specimen. The internal standard for the metabolite analysis was then added (20 μl of a 0.25-mg U-11,824 per ml solution in methanol) using a BDL positive displacement pipettor (Becton Dickinson Labware, Oxnard, CA, U.S.A.). After using the syringe action of the pipettor to mix the barrel contents, vacuum (660 mmHg internal manifold pressure) was applied and the eluent was discarded. The columns were washed with 0.5 ml water, twice (discarding the eluent), and aspirated for 1 min to remove excess water. The C_{18} columns were then removed from the vacuum manifold and replaced with silica gel columns, to the top of which were attached the loaded C_{18} columns using a special column-to-column adaptor (Analytichem International). The drug and metabolites were then eluted by vacuum aspirating two 0.5-ml washes of methanol through the connected columns. The methanol eluent was collected in 10 × 75 mm glass culture tubes and evaporated to dryness at 40°C under a stream of nitrogen. The resulting residue was reconstituted in 0.5 ml of 40% methanol by first adding 0.2 ml of a methanolic solution containing the internal standard for the ciglitazone analysis (2 μg of U-53,059 per ml in methanol) and vortexing for 45 sec, followed by adding 0.3 ml of water and vortexing for 15 sec. This reconstitution procedure

Sample Prep Procedure

A. Load Sample Onto C-18 BOND ELUT™ Column



B. Elute Analytes Through a Silica BOND ELUT™ Column



C. Evaporate and Reconstitute for Assay

1. Evaporate under N_2 at 40°C
2. Add 0.2 ml methanol I.S. solution (U-53,059) and vortex
3. Add 0.3 ml water and vortex

Fig. 5. Flow diagram of the serum extraction and cleanup procedure.

was necessary to ensure complete solubilization of the drug and metabolites. Using this extraction/cleanup procedure, summarized in Fig. 5, ten serum samples could be processed simultaneously in less than 1 h.

Standard preparation

Serum standards were prepared from pooled, blank serum specimens obtained from human volunteers. Accurately weighed amounts of ciglitazone and the four metabolite standards were dissolved in methanol to produce a stock concentration of 1 mg/ml for each metabolite and 0.5 mg/ml for ciglitazone. This solution was serially diluted with methanol to produce standard working solutions. Aliquots (10 ml) of fresh blank serum were then spiked with the standard solutions, keeping the methanol concentration of the resulting serum standards less than 2% (v/v). (This procedure produced more reliable results than were obtained by evaporating the standard solutions in a

vial and reconstituting with serum because of the limited solubility of ciglitazone in aqueous media.) After mixing, the serum standards were dispensed in 0.7-ml aliquots and frozen at −20° C until the time of analysis. Stability studies indicated no decline in potency after two weeks of storage.

Quantitation method

For quantitation purposes, the analysis for ciglitazone and its metabolites was treated as two independent assays, each having its own analytical system and internal standard. The internal standard (I.S.) for ciglitazone was U-53,059. The internal standard for the metabolites was U-11,824. Average response factors for each component were determined by analysis of serum standards that had been extracted and processed identically to unknown serum samples. The response factor for ciglitazone was calculated as the peak height ratio (ciglitazone/I.S.) divided by concentration, and the response factors for the metabolites were calculated as the peak area ratio (metabolite/I.S.) divided by concentration. The serum samples from each subject given the drug were analyzed as a set. Typically, each set consisted of fiteen samples and was analyzed with five serum standards having component concentrations dispersed evenly over the concentration range expected in the unknowns. The component concentrations in the unknowns were calculated by dividing the observed peak height or area ratio by the corresponding average response factor.

In vivo studies

Apparently healthy non-diabetic adult male volunteers were fasted 10 h prior to treatment and for 4 h following administration of four 250-mg compressed tablets of ciglitazone. Blood samples were collected at predetermined times ranging from 0 to 168 h post-dose. Serum was prepared as described above and stored frozen at −20° C.

RESULTS AND DISCUSSION

Chromatography

An isomodal column switching technique is described that permits the simultaneous quantitation of ciglitazone and its monohydroxyl metabolites by isocratic, reversed-phase HPLC (UV). The HPLC system consists of two isocratic units linked together via a common guard column (Fig. 3). The isocratic systems employ the same type of analytical column (C_{18}, 5 μm, porous particle), but use different strengths of mobile phase: system A is 40% acetonitrile; system B is 70% acetonitrile. The serum extract is injected with the guard column in-line with system A (Fig. 4). After the more polar components (metabolites and their internal standard, U-11,824) have cleared the guard column and been routed onto analytical column A, the guard column is switched in-line with system B for the analysis of the less polar components (ciglitazone and its internal standard, U-53,059). When the analysis on system A is complete, the guard column is switched back in-line with the weaker mobile phase to re-equilibrate for the next injection. The total run/recycle time for each injection is 25 min, and the column switching is done automatically with air-actuated valves and an event timer. The technique is called isomodal

because the entire analysis is conducted using one chromatographic mode (reversed-phase C_{18} columns with aqueous acetonitrile mobile phase).

The success of the system depends on the large difference in capacity factors between the parent drug and metabolites on the C_{18} guard column. Using mobile phase A, the metabolites and U-11,824 cleared the guard column in < 4 ml elution volume (determined by connecting the guard column directly to the detector). Under the same conditions, the elution volume of ciglitazone is 40 ml. Thus, at the 5-min run-time (7.0 ml elution volume) when the guard column is switched in-line with system B, the metabolites have completely eluted from the guard column while ciglitazone is fully retained. Since this separation is crucial for column routing, the ruggedness of the analytical system depends heavily on the ruggedness of the guard column. Using the Brownlee 3-cm Spheri-5 cartridge, it appears that a conservative estimate of column lifetime is 300 injection cycles. Guard column fatigue is detected as peak broadening or asymmetry rather than failure to retain ciglitazone. Typical component retention times and within-run relative standard deviations are given in Table I. Representative chromatograms of solution standards are shown in Fig. 6. Good system stability was indicated by retention time relative standard deviations for all components of $\leqslant 0.2\%$. The analytical columns exhibited approximately 12,000 theoretical plates for each component (N is uncorrected for peak skew). All components had peak asymmetry factors between 1.3 and 1.5 (ciglitazone = 1.4). Similar values were obtained when ciglitazone was analyzed on system B alone, indicating that the delayed elution with column switching did not adversely affect the chromatography. A relatively large injection volume (0.2 ml) was used because of autosampler requirements, but it was found that the 40% methanol sample solvent did not

TABLE I

ANALYTE RETENTION TIMES AND WITHIN-RUN RETENTION TIME PRECISION

Analyte name	Chromatographic system routing	Retention time* ± S.D. (min)	Relative standard deviation (%)
Trans-4′-ol	A	9.19 ± 0.02 (n = 20)	0.2
Cis-3′-ol	A	12.09 ± 0.02 (n = 20)	0.2
Trans-3′-ol	A	12.53 ± 0.01 (n = 9)	0.1
Cis-4′-ol	A	13.48 ± 0.02 (n = 20)	0.2
U-11,824 (I.S.)	A	17.98 ± 0.02 (n = 20)	0.1
Ciglitazone	B	17.07 (12.07)** ± 0.02 (n = 20)	0.2
U-53,059 (I.S.)	B	22.68 (17.68)** ± 0.03 (n = 15)	0.1

*Time reckoned from time 0-min injection.

**Parenthetical values are the retention times in system B reckoned from the time 5-min injection of the guard-column retentate.

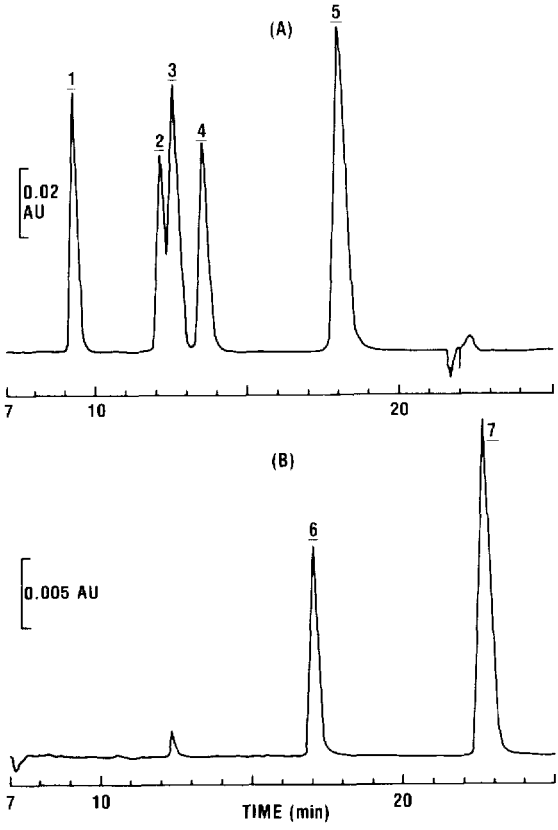


Fig. 6. Simultaneous chromatograms collected during the analysis of a solution standard containing approximately 5 μg/ml of each metabolite (A) and 0.5 μg/ml of ciglitazone (B). Peaks: (1) *trans*-4′-ol; (2) *cis*-3′-ol; (3) *trans*-3′-ol; (4) *cis*-4′-ol; (5) U11,824, I.S.; (6) ciglitazone; and (7) U-53,059, I.S. See text for chromatographic conditions.

contribute to band broadening when injected with mobile phase A (40% acetonitrile). All components were baseline resolved with the exception of *cis*-3′-ol and *trans*-3′-ol (resolution factor (R_s) = 0.9).

During the development of this method, other columns (silica, CN, TMS, C_8) were tested for their ability to compress the drug/metabolite separation into a single isocratic chromatogram. None was successful, nor did any of the columns resolve the *trans*-3′-ol and *cis*-3′-ol metabolites as well as the C_{18} column. Maximum resolution of these components was obtained with a C_{18} column and a mobile phase consisting of $\leqslant$ 40% acetonitrile at a flow-rate of $\leqslant$ 1.5 ml/min. (Acetonitrile was comparable to methanol for achieving this separation, and considerably better than tetrahydrofuran.) The pH of the mobile phase was adjusted to 2.5 to suppress ionization of the thiazolidinedione functional group (pK_a = 5.7).

Using a mobile-phase strength that gave acceptable capacity factors for the metabolites (k' = 3—6), the retention time of ciglitazone on the C_{18} column was greater than 2 h (k' = 40). This made it impractical to perform the analysis by isocratic elution. Torii et al. [17] overcame this problem by using thin-layer chromatography to separate the serum extract into parent drug and metabolite

fractions and then subsequently analyzing them with separate isocratic HPLC systems. Isomodal column switching techniques [6—10] offer a convenient alternative in this situation; as described here, the serum extract is fractionated into polar and non-polar components for subsequent isocratic HPLC analysis by an automated, on-line system. This approach combines the simplified sample workup used for gradient HPLC with the stability and sensitivity of isocratic HPLC, and is also relatively fast (step gradient run/recycle time of 45 min for this separation compared to the column switching run/recycle time of 25 min).

Serum extraction and percentage recovery

The serum extraction procedure was designed for rapid, one-step sample cleanup of both ciglitazone and its monohydroxyl metabolites. After buffering to pH 7.0, the serum was drawn through a C_{18} Bond Elut column (Fig. 5). The column was washed with water and then connected in series with a Bond Elut silica gel column, which was used to remove several polar components that interfered with metabolite quantitation. The analytes were eluted from both columns with two washes of methanol. The methanol was then evaporated and the extract was reconstituted for analysis.

Using this procedure, the recovery of the drug and metabolites from aqueous

TABLE II

PERCENTAGE RECOVERY OF CIGLITAZONE AND METABOLITES FROM HUMAN SERUM

Serum concentrations ranged from 0.1 to 20 μg/ml for the metabolites and 0.05 to 10 μg/ml for ciglitazone. Recovery was independent of concentration.

Analyte name	Parameter	Percent recovery			Pooled estimate of percent recovery
		Day 1	Day 2	Day 3	
Trans-4'-ol	$\bar{X}$	80.1	81.4	82.3	81.2
	S.D.	5.7	3.1	3.9	4.1
	R.S.D.	7.1	3.8	4.7	5.1
	n	8	8	8	24
Cis-3'-ol	$\bar{X}$	85.1	84.5	89.3	86.3
	S.D.	4.8	3.6	2.1	4.1
	R.S.D.	5.7	4.2	2.4	4.8
	n	8	8	8	24
Trans-3'-ol	$\bar{X}$	91.3	90.9	93.8	92.0
	S.D.	4.2	2.2	2.7	3.3
	R.S.D.	4.6	2.4	2.9	3.5
	n	8	8	8	24
Cis-4'-ol	$\bar{X}$	88.7	94.7	92.5	91.8
	S.D.	4.6	5.7	2.6	4.9
	R.S.D.	5.2	6.0	2.8	5.4
	n	7	7	7	21
Ciglitazone	$\bar{X}$	60.1	62.7	63.7	62.1
	S.D.	6.7	3.7	3.5	5.0
	R.S.D.	11.2	5.9	5.6	8.0
	n	8	8	8	24

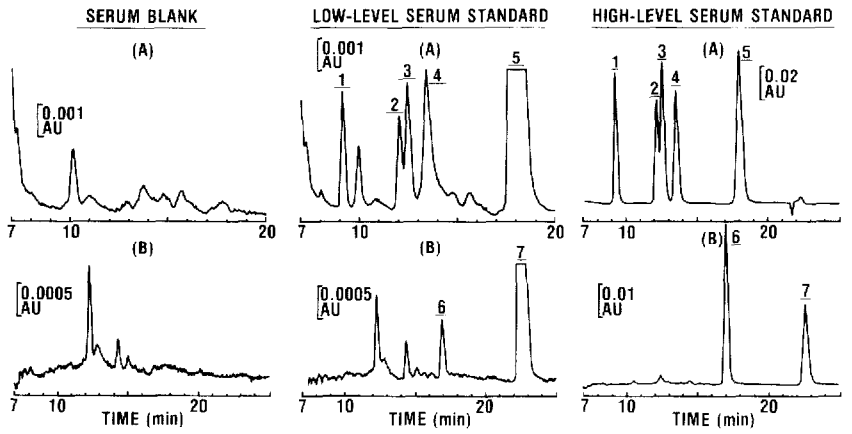


Fig. 7. Simultaneous chromatograms collected during the analysis of serum standards for metabolites (A) and ciglitazone (B). The low level standard was spiked with roughly 0.2 μg/ml of each metabolite and 0.1 μg/ml of ciglitazone; the high-level standard was spiked with 5 μg/ml of each metabolite and 2.5 μg/ml of ciglitazone. Peak identification as in Fig. 6.

solutions was quantitative. From serum, however, the absolute recovery was lower: 81—92% for the four metabolites and 62% for ciglitazone (Table II). The recovery of each component was determined at eight concentrations ranging from 0.1 to 20 μg/ml for the metabolites and 0.05 to 10 μg/ml for ciglitazone. Over this concentration range, the percent absolute recovery for each component was constant with a relative standard deviation of roughly 5% for the metabolites and 8% for ciglitazone (Table II, representative chromatograms are shown in Fig. 7). Additional methanol washes did not improve the ciglitazone recovery. Protein binding studies have shown the drug to be strongly bound (> 95% in plasma) [17] and this may interfere with the bonded-phase extraction process. Serum aging studies with serum standards indicated that there was no change in analyte absolute recovery from samples stored at −20°C for four weeks.

TABLE III

LINEARITY AND REPRODUCIBILITY OF SERUM STANDARD CALIBRATION CURVES

Serum standard concentrations ranged from 0.1 to 20 μg/ml for the metabolites and from 0.05 to 10 μg/ml for ciglitazone.

Parameter	*Trans*-4′-ol			*Cis*-3′-ol		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Mean response factor* (X 100)	9.57	9.16	9.20	9.81	9.34	9.52
±S.D.	0.38	0.24	0.40	0.31	0.32	0.38
R.S.D.	3.9	2.7	4.3	3.2	3.5	4.0
Slope**	0.0967	0.0930	0.0929	0.0992	0.0946	0.0956
y-Intercept***	0.0023	−0.0027	0.0022	0.0001	0.0011	−0.0034
r	>0.999	>0.999	>0.999	>0.999	>0.999	>0.999

*Response factor = (peak area of analyte)/[(peak area of internal standard) X (concentration of analyte in μg/ml)].

**Obtained from best-fit linear regression analysis of a plot of peak height or area ratio (analyte/internal standard) versus the analyte concentration in μg/ml.

***The *y*-intercept was insignificant ($P > 0.05$) in all cases.

Internal standards

Since the analytical method employs two isocratic HPLC systems for the analysis of ciglitazone and its metabolites, two internal standards are required for quantitation. U-11,824 was found to be a suitable internal standard for the quantitation of the metabolites. It elutes in a region of the metabolite chromatogram free from interferences (see Fig. 7), it is extracted with similar efficiency from serum by a C_{18} extraction column (80% recovery), and factors that cause a change in the metabolite recovery also cause a similar change in U-11,824 recovery. It was therefore added to the serum prior to extraction.

Conversely, a suitable internal standard to control for the extraction variability of ciglitazone could not be located. Several sulfonylureas and related compounds were tested, but all had either unacceptably low extraction recovery (< 50%) or improper retention time. As a consequence, U-53,059 was selected as a chromatographic internal standard and was added during reconstitution of the extract for analysis.

Linearity

Data on the linearity and reproducibility of serum standard calibration curves on three separate days are given in Table III. The response factors for the metabolites were constant over the range 0.1—20 μg/ml, with relative standard deviations of roughly 4%. Plots of peak area ratio versus concentration for all of the metabolites had insignificant *y*-intercepts and correlation coefficients of ⩾ 0.999. The response factor for ciglitazone was also constant over the concentration range 0.05—10 μg/ml, with a relative standard deviation of roughly 8%. Plots of peak height ratio versus concentration had insignificant *y*-intercepts and correlation coefficients of ⩾ 0.994. The larger relative standard deviation and poorer linear correlation coefficient for ciglitazone compared to the metabolites are probably due to the poorer ciglitazone extraction efficiency and to the lack of an internal standard to account for ciglitazone extraction variability (U-53,059 is a chromatographic internal standard only). A minor increase in precision was obtained by using U-11,824 as the internal standard for both assays, but the improvement was not sufficient to justify the additional manual calculations required by limitations of the computer system.

Trans-3′-ol			*Cis*-4′-ol			Ciglitazone		
Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
11.8	11.2	11.4	10.6	10.4	10.2	76.4	80.6	81.3
0.4	0.4	0.4	0.5	0.5	0.3	8.6	4.8	4.5
3.4	3.3	3.4	4.4	5.2	2.7	11.2	5.9	5.6
0.115	0.110	0.112	0.106	0.103	0.102	0.855	0.796	0.793
0.005	0.006	0.004	0.004	0.007	0.000	−0.133	0.014	0.059
>0.999	>0.999	>0.999	0.999	>0.999	>0.999	0.994	0.998	0.998

Precision and accuracy

The intra-day and inter-day precision and accuracy of the assays were evaluated at three different concentration levels for each metabolite and ciglitazone by repeating triplicate assays of spiked serum samples on three days. The results of these experiments are presented in Table IV. The mean intra-day relative standard deviation for the assay over the concentration range 0.2—5 μg/ml for the metabolites and 0.1—2.4 μg/ml for ciglitazone appears to be roughly 6%. There appears to be no significant inter-day variation of assay results over this concentration range. There also appears to be no significant assay bias, since pooled estimates of assay error for both ciglitazone and the metabolites were (with one exception) less than ± 4%.

TABLE IV

ASSAY PRECISION AND ACCURACY

Serum samples were analyzed in triplicate on each of three successive days in order to determine both intra- and inter-day method variance. Analysis of variance procedures ($P > 0.05$) indicated that there was no significant interday difference in assay results for any of the analytes, and so the data for all three days ($n = 9$) were pooled.

Analyte name	Actual concentration of spiked serum of sample (μg/ml)	Mean assay result ($n = 9$) ± S.D. (μg/ml)	Relative standard deviation (%)	Percent error*
Trans-4'-ol	5.03	5.06 ± 0.29	5.7	0.6
	0.503	0.500 ± 0.020	4.0	−0.6
	0.202	0.195 ± 0.008	3.9	−3.5
Cis-3'-ol	4.55	4.68 ± 0.28	6.0	2.9
	0.455	0.466 ± 0.011	2.4	2.4
	0.182	0.181 ± 0.009	5.0	−0.5
Trans-3'-ol	6.21	6.33 ± 0.32	5.0	1.9
	0.621	0.645 ± 0.013	2.0	3.9
	0.249	0.257 ± 0.014	5.6	3.2
Cis-4'-ol	5.51	5.51 ± 0.27	5.0	0.0
	0.551	0.555 ± 0.019	3.4	0.7
	0.221	0.209 ± 0.012	5.7	−5.4
Ciglitazone	2.37	2.37 ± 0.09	3.7	0.0
	0.237	0.230 ± 0.015	6.7	−3.0
	0.095	0.094 ± 0.006	6.7	−1.4

*Percent error = [(observed − actual)/actual] × 100.

Sensitivity

The assay quantitation limit was defined as that concentration of analyte which produces a peak roughly ten times the size of serum blank peaks having retention times such that they may interfere with analyte measurement. The assay quantitation limit for the metabolites was roughly 0.1 μg/ml. The quantitation limit for ciglitazone was 0.05 μg/ml.

Application

The utility of the assay method was demonstrated by analyzing serum

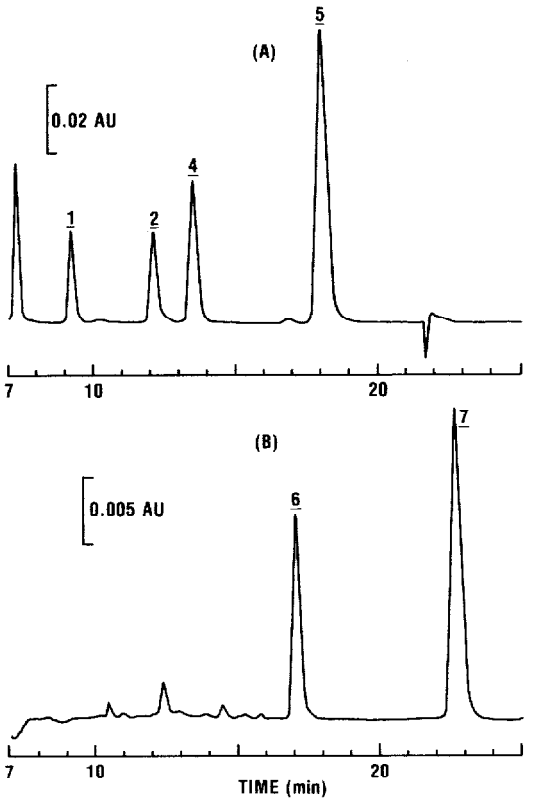


Fig. 8. Simultaneous chromatograms collected for the metabolites (A) and ciglitazone (B) during the analysis of a 3-h post-dose serum sample from a human subject given a single 1000-mg oral dose of ciglitazone. Peak identification as in Fig. 6.

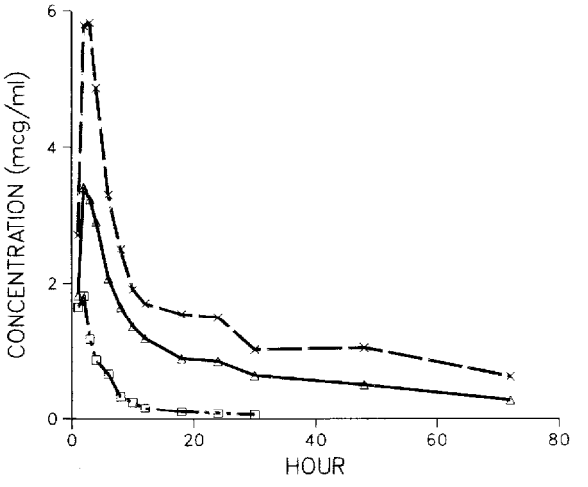


Fig. 9. Composite serum concentration—time profile from three human subjects given single 1000-mg oral doses of ciglitazone. (△), *cis*-3′-ol; (×), *cis*-4′-ol; and (□), ciglitazone.

samples from selected non-diabetic, human male volunteers participating in a ciglitazone phase I tolerance study. Based on coelution with authentic standards, measurable levels of ciglitazone, *cis*-4′-ol, *cis*-3′-ol, and *trans*-4′-ol were detected in serum following a 1000-mg oral dose (Fig. 8). The *trans*-3′-ol metabolite was not detected. (In order to form a distinct shoulder on the *cis*-3′-ol peak, the *trans*-3′-ol concentration must be > 20% of the concentration of the *cis*-3′-ol.) As shown in the composite serum concentration—time profile (Fig. 9), the assay method was sufficiently sensitive to assay the 78-h timepoint for the metabolites (2—3 times the $\beta t_{1/2}$) and the 30-h timepoint for ciglitazone (3 times the $\beta t_{1/2}$). Selected timepoints were assayed in duplicate and found to agree within the estimated method relative standard deviation.

CONCLUSION

The applicability of an isomodal, column switching HPLC technique was demonstrated for the routine, quantitative analysis of ciglitazone, a potential oral diabetic agent [1, 2], and its monohydroxyl metabolites in human serum. This analysis could not be conducted using a simple isocratic HPLC technique because of the polarity difference between ciglitazone and the metabolites. Resolution of the metabolite isomers (*cis*- and *trans*-isomers of 3′- and 4′-hydroxy metabolites) required a reversed-phase C_{18} HPLC system, on which the capacity factor ratio of the parent drug to metabolites was approximately ten to one. Rather than perform the analysis by gradient elution HPLC, an HPLC apparatus was designed to conduct on-line fractionation of the drug/metabolite serum extract into polar (metabolite) and non-polar (drug) fractions that were then automatically routed into individually optimized, isocratic, reversed-phase systems for analysis.

This column switching procedure performs essentially the same separation that could have been accomplished by a reversed-phase step gradient technique or by manually dividing the sample into equal portions for analysis on separate, reversed-phase, isocratic systems. The separation selectivity of all these methods is the same because they all rely on a single, identical, chromatographic mode. The advantage of the isomodal column switching approach over the manual two-injection procedure is that it conducts the same separation automatically and with a single sample injection. Compared to the gradient method, the column switching procedure is faster (25-min run/recycle time for column switching compared to 45-min run/recycle time for gradient), and it has the reliability and stability of isocratic elution methods.

For more general applications, this approach to multicomponent analysis could, in principle, be used with any chromatographic mode for which there is a large difference in capacity factors between the drug and metabolites. In this respect, isomodal column switching is equally versatile to gradient elution. A serious limitation of this column switching approach is that unlike gradient elution, which permits analysis for multiple analytes with uniformly distributed capacity factors over a broad range, the column switching method is restricted to the analysis of samples in which the analytes fall into one of two relatively narrow capacity factor ranges. For situations that meet this requirement, the isomodal column switching technique offers advantages over conventional

analysis procedures in terms of speed and reliability for routine, quantitative, drug/metabolite assays.

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