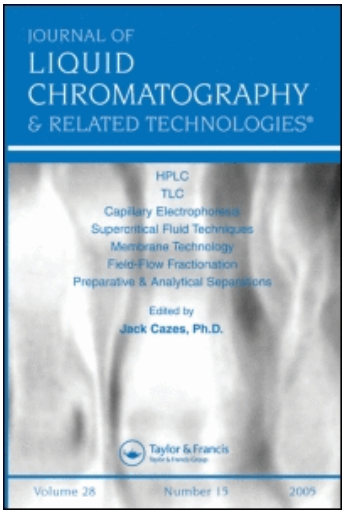

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HPLC ASSAY OF A LEUKOTRIENE D_4 ANTAGONIST IN PHARMACEUTICAL PREPARATIONS AND BIOLOGICAL SAMPLES WITH UV AND FLUORESCENCE DETECTION

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ABSTRACT

Analytical methodology has been developed to assay L-660,711, 3-(((3-(2-(7-chloro-2-quinolinyl)ethenyl)phenyl)((3-dimethylamino-3-oxopropyl)thio)methyl)thio)propionic acid to assess drug stability in pharmaceutical preparations and drug concentration in plasma samples. Reversed-phase HPLC chromatography with UV detection at 232 nm and 300 nm was used to analyze drug content and assess stability in pharmaceutical formulations. In addition to UV, fluorescence detection with 300 nm and 400 nm as the excitation-emission wavelength pair was used to enhance sensitivity for biological samples with low drug concentrations. The latter system had a detection limit of 1-5 ng/ml for a 25 µl injection. It was used to monitor drug concentration in plasma following intrapulmonary, intravenous and oral administration.

INTRODUCTION

The compound, 3-(((3-(2-(7-chloro-2-quinolinyl) ethenyl)phenyl)((3-dimethylamino-3-oxopropyl)thio)methyl)thio)propionic acid

is a potent leukotriene antagonist undergoing clinical trials. This drug is active by the intravenous, oral and intrapulmonary routes of administration against leukotriene-induced and allergen-induced bronchoconstriction in various biological models (1).

The evaluation of pharmaceutical preparations involves many considerations which include the chemical stability and bioavailability of the drug. This contribution describes the analytical methods used to evaluate chemical stability in some pharmaceutical formulations and to measure drug concentrations in biological samples to evaluate various pharmacokinetic parameters as a function of route of administration and formulation characteristics. The analytical methods presented here involve two differnt chromatographic methods for the separation of the drug from endogeneous molecules and two detection systems that, in conjunction with the chromatographic separation, afford different detection limits and selectivities. These methods have been successful in assaying drug concentrations in plasma or serum collected from rats, guinea pigs, dogs, sheep and monkeys. A separate publication will discuss in more detail some aspects of the pharmacokinetic behavior of this compound.

MATERIALS AND METHODS

Equipment

The high performance liquid chromatography unit (HPLC) used to assay pharmaceutical preparations consisted of an HP1090 equipped with an automatic injector, a column oven, a diode array UV detector, a DPU board, a 10 mb hard disk and a HP85B computer (Hewlett Packard Avondale, PA 19311).

The HPLC used for biological samples consisted of Waters equipment: (division of Millipore, Milford, MA 01757) a model 510 pump, a WISP 712, a LambdaMax 481 UV detector and a HP3392A

integrator. The column temperature for the biological assay was controlled with a Thermometrix 1419 (B. Braun, Melsungen AG, W. Germany) and an insulated water bath. The fluorescence equipment consisted of a Perkin Elmer (Norwalk CT 06856) luminescence spectrometer LS5 with a Perkin Elmer 3600 data station and either a LC cell accessory or a standard turret cell holder.

The UV spectrometer used to record solution spectra was a HP8451A diode array.

Chromatographic Separation

Two different mobile phases and columns were used that afforded different selectivities, peak symmetries and sensitivities.

1. The first set of conditions consisted of a 12.5:12.5:75 (v:v:v) mixture of tetrahydrofuran (THF), 1-propanol and 0.2% (w/v) aqueous phosphoric acid, respectively, with a 10 cm Spherisorb Octyl column containing 3 µm particles. The flow rate was 2 ml/min with a column temperature of 50°C.
2. The second set of conditions consisted of a 47.5:52.5 (v:v) mixture of 0.02M phosphate (pH 7.2) and methanol, respectively. The flow rate was 2 ml/min and temperature was 45°C. The HPLC column was 10 cm long, filled with 3 µm Spherisorb ODS2.

Reagents

All the reagents used were of analytical or HPLC grade unless otherwise noted. Due to impurities present in regular analytical grade THF and 1-propanol, preservative free THF (BDH

Canada, Montreal, Canada) and high purity 1-propanol (Burdick and Jackson, division of American Hospital Supply Co., McGraw Park, IL 60085) solvent were used.

The drug, L-660,711, and other structurally-related compounds were prepared by the Medicinal Chemistry group at Merck Frosst Canada Inc. (Pointe Claire-Dorval, PQ).

Preparation of the Biological Samples

The blood samples were drawn with either an heparinized Vacutainer (Becton and Dickinson, Orangeburg, NY 10962) or a syringe. The blood collected with a syringe was immediately transferred to a heparinized test tube. The plasma was collected after centrifugation (3000 RPM for 15 min) in a IEC Centra-7C refrigerated centrifuge (International Equipment Co., a division of Damon, Needham Hts, MA 02194). Blood samples from monkeys were allowed to clot for 20 min in test tubes and centrifuged to collect the serum.

The serum or plasma was processed by adding two volumes of methanol to one of sample and followed by centrifugation in an Eppendorf 5415 centrifuge (Brinkmann Instruments, Inc, Westbury NY 11590) at 14,000 RPM for 10 min. The supernatant was transferred to a 300 µl PolySpring Glass Insert located in an amber vial and stoppered with a Teflon lined cap and septum (National Scientific Co., Norcross, GA 30093). Standards were prepared by spiking the plasma or serum with known quantities of an aqueous solution of the drug as the sodium salt.

Detection and Quantitation of Pharmaceutical Samples

The pharmaceutical preparations were analyzed using the first set of assay conditions described above. Two detection wavelengths, 232 and 300 nm, with a band width of 4 nm were used to monitor peak areas. The first wavelength was selected to monitor all UV absorbing species. The second wavelength was used to determine the presence of broad band absorbers such as

quinoline-containing degradation products. The ratio of both was used to identify peak overlap.

The standard consisted of a methanol solution of the free acid. A conversion factor ($M_{w,Na}/M_{w,H}$, 537.1/515.1) of 1.0427 was used to quantitate the sodium salt. The typical column performance provided efficiences of 6500-5500 plates per column for L-660,711. Columns that reached the lower limit were replaced. Inconsistent performance was found with columns stored in the mobile phase. Routinely, columns were stored in a 30:70 methanol-water mixture.

The linearity of the method was established for the concentration range of 0.1-0.4 mg/ml (correlation coefficient = 0.999), with zero intercept ($p<0.05$), for injection volumes of either 2 or 5 µl. The relative standard deviation of multiple injections ($n>15$) was less than 1%. This concentration range corresponded to that used in the preparation of the pharmaceutical stability samples. The upper value was limited by the solubility of the drug in methanol. Lower concentrations were not investigated because large nominal concentrations were necessary to identify minute amount of decomposition products. Larger concentrations of drug in alkaline buffer have been injected at the expense of more distorted peaks and column inlet void formation.

Optimum reproducibility and reliability were obtained with mobile phases that were thoroughly degassed to avoid gas bubbles forming in the detector. Degassing by using vacuum or by bubbling an inert gas should be avoided to prevent evaporation of the lower boiling solvent, THF. Best results were obtained after sonication of the mobile phase.

Detection and Quantitation of Biological Samples

Primarily biological Samples were assayed using the first set of chromatographic conditions described (see Chromatographic Separation) and a 300 nm UV detection wavelength. This

wavelength was selected for optimum detectability and selectivity considering interferences caused by early eluting peaks. The 300 nm wavelength allowed the identification of any quinoline-containing metabolites which might have been present.

Fluorescence detection was used to enhance sensitivity for biological samples containing low drug concentrations. The fluorescence detector was optimized for signal-to-noise-ratio for both separation systems. In both cases, a large number of wavelength pairs (excitation and emission) were found to be equally suitable for the assay. The 300 nm and 400 nm excitation-emission wavelength pair was chosen for selectivity after preliminary evaluation of the baseline following injection of plasma samples. The slit width was optimized at 15 and 10 nm for the excitation and emission, respectively, for this wavelength pair using the same approach.

Several pharmacokinetic studies (greater than 30 studies) were performed with either separation and detection systems described above. In each case, a calibration curve was established with a minimum of three injections of the standard sample at four levels of concentration. Each standard was prepared by spiking plasma or serum collected from each animal prior to dose administration. All calibration curves had correlation coefficients larger than 0.99 and a zero intercept ($p<0.05$). The slopes of the calibration curves were reproducible ($p<0.05$, RSD 3-5%). If a calibration curve did not meet these criteria, appropriate measures were taken to correct the HPLC chromatographic conditions.

Each new column was tested by injecting 25 µl of a biological sample. Under these conditions, column efficiencies were about 5000 plates. The column was replaced before 3500 plates were obtained. This plate count criterion was always met before column pressure or variable retention time became a problem. The normal life time of a column was about 700 injections and no attempts were made to restore disabled columns.

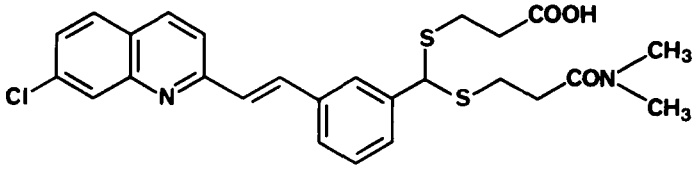
RESULTS AND DISCUSSION

The compound L-660,711 (see Fig 1 for structure) has been prepared as the free acid or the sodium salt. The free acid is a free flowing powder, nonhygroscopic and sparingly water soluble in unbuffered solutions. The sodium salt is more soluble in aqueous solutions (pH>7.5) but very hygroscopic.

Spectroscopic Characteristics

The effect of pH on the UV absorption of L-660,711 is shown in Fig 2. The spectrum obtained with the compound dissolved in a neutral or alkaline solution was not significantly different (see spectra b and c of Fig 2). The spectrum was significantly different in the acidic environment. The spectrum was the same as that observed for solutions of neutral pH when organic modifiers were added, irrespective of the initial pH of the solution. These observations indicated that an important change in the resonance structure of the chromophore occurred with the protonation at the N-quinoline in an acidic medium.

Fig 3 shows the effect of pH on the emission spectrum at an excitation wavelength of 300 nm. Fig 4 shows the effect of pH on the emission spectrum at an excitation wavelength of 250 nm. In aqeuous solutions, a maximum emission was found at neutral pH. The fluorescence intensities were increased substantially in the presence of organic modifier irrespective of the initial aqueous pH values.



L-660,711

FIGURE 1 The chemical structure of L-660,711 as the free acid.

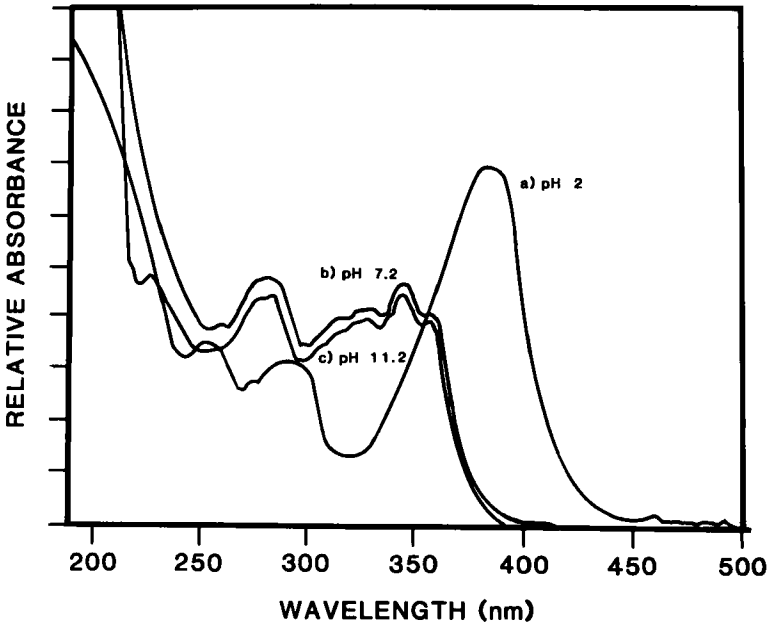


FIGURE 2 The effect of pH on the UV spectrum of L-660,711 (1.6 µg/ml). The solutions were prepared in 0.02M phosphate adjusted to a) pH 2.0, b) pH 7.2, c) pH 11.2.

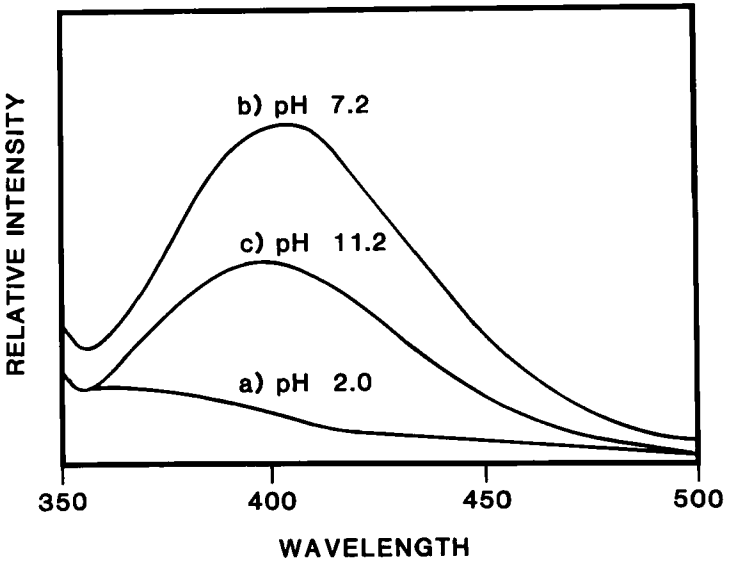


FIGURE 3 The effect of pH on the fluorescence spectrum of L-660,711. The excitation wavelength was 300 nm. Both the excitation and emission slits were 10 nm.

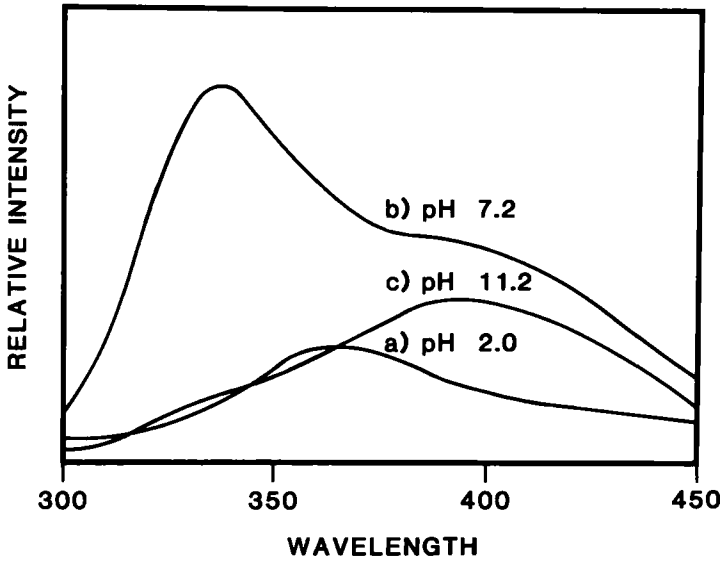


FIGURE 4 The effect of pH on the fluorescence spectrum of L-660,711. The excitation wavelength was 250 nm. Both the excitation and emission slits were 10 nm.

Assay and Stability of the Pharmaceutical Preparations

Good resolution of drug, transformation products and potential process impurities is an important criterion for HPLC assays developed to analyze pharmaceutical formulations. The major transformation product for L-660,711 was its cis isomer resulting from photochemically-induced isomerization. Fig 5 shows a chromatogram of photodecomposed L-660,711 obtained at detector wavelengths of 232 nm and 300 nm showing the resolution of the trans isomer (retention time: 5.9 min) and its phototransformation product the cis isomer (retention time: 4.2 min). Other minor degradation or transformation products have not been identified. Potential process impurities, other than the cis isomer, include the diacid and the Michael addition product.

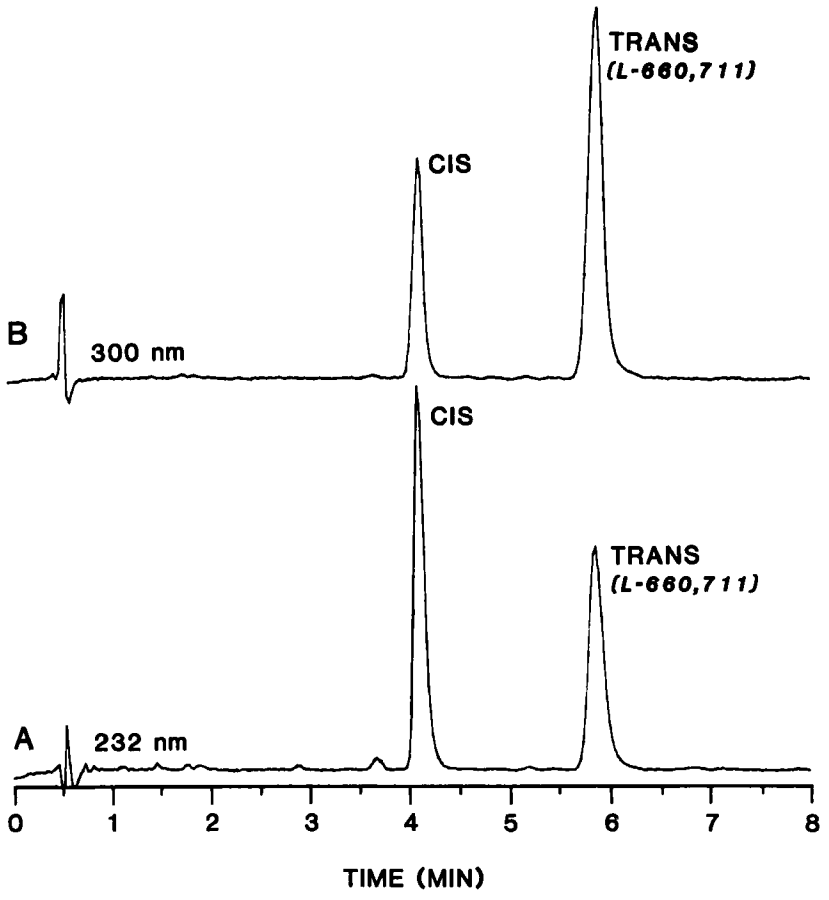


FIGURE 5 The chromatographic separation of the transformation products after exposing samples for two days to ambient light. The drug and the cis isomer retention times were 5.9 min and 4.2 min, respectively. Diode array detection was at a) 232 nm and b) 300 nm. See the text for chromatographic conditions.

Aqueous solutions of the drug were tested for stability in the presence of fluorescent light (30°C and 355 footcandles,FC) and in the absence of light (30°C). Table 1 shows that drug stability, in the presence of light, is concentration dependent and short term. The cis isomer is the major phototransformation product. Table 2 shows that, when protected from light, the compound was stable for at least 7 days in aqueous solution. Some decomposition products were found at 20 days and 37 days. Similar results were obtained with methanolic solutions.

The neat drug, both as the free acid and the sodium salt was stable in the solid state at 50°C for at least 12 months.

Assay of Plasma Samples

This drug has been administered to animals via the intravenous, oral and aerosol routes. The determination of the concentration-time profile of the drug resulting from these experiments involved the determination of plasma concentrations in the range of concentration of 1 ng/ml-100 μg/ml. Both peak area and height were used for the quantitation of biological samples. Peak height was preferred near the detection limit since this mode of quantitation was less susceptible to interferences from adjacent peaks.

The first set of HPLC conditions was used to analyze for drug concentrations in plasma after IV or oral drug administration. With the UV detector set at 300 nm and a 25 μl injection, the detection limit was about 0.5 μg/ml (3 standard deviations). An example of data obtained when a 5 mg/kg IV dose was given to four beagle dogs is shown in Fig 6.

An order of magnitude improvement in detection limits was obtained with fluorescence detection. The method, using either UV or fluorescence detection, satisfied the validation criteria described in the section entitled Detection and Quantitation of Biological Samples. The calibration curves prepared for each assay were in the range of concentration 0.7 - 20 μg/ml and

TABLE 1

Stability of aqueous solutions of 1.25% $NaHCO_3$ (pH 8.3) at 30°C when stored under fluorescent light at 355FC. Stability results are reported as percent of the assay results obtained from the initial samples.

CONCENTRATION (mg/ml)	TIME			
	1 hour	4 hours	8 hours	24 hours
0.1	77	18	16	10
1.0	74	21	22	6
10.0	97	80	65	32

TABLE 2

Stability of aqueous L-660,711 solutions in 1.25% $NaHCO_3$ (pH 8.3) at 30°C when protected from light. The lower two concentrations were injected (triplicates) directly into the chromatograph while the largest concentration was prepared in triplicate with a 40 fold dilution before injection. Stability results are reported as percent of the assay results obtained from the initial samples. Standard deviation for each determination was 2 (n=3 for the first two rows and n=9 for the remainder).

CONCENTRATION (mg/ml)	TIME				
	1 day	2 days	7 days	20 days	37 days
0.1	100.4	100.0	102.0	97.5	96.0
1.0	100.7	100.0	99.2	96.7	93.1
10.0	103.0	103.0	102.9	99.4	98.0

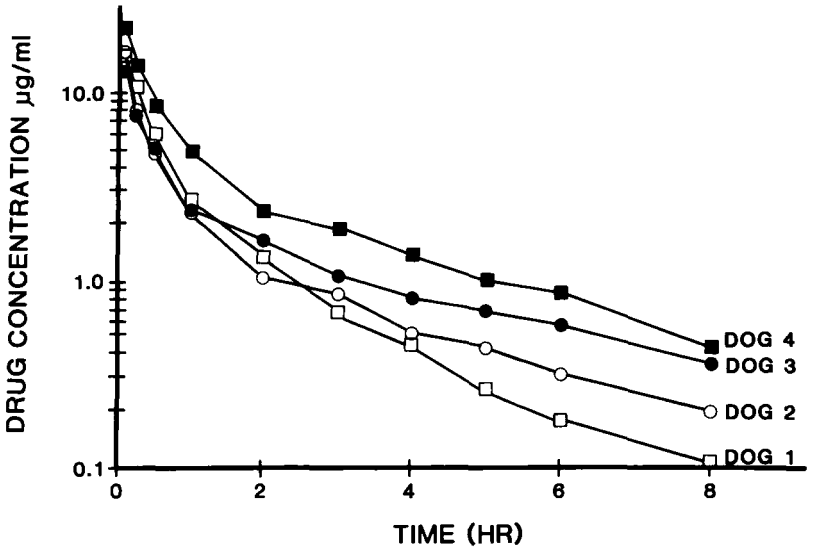


FIGURE 6 Drug-concentration profiles after a 5 mg/kg IV dose was given to four dogs weighing 10 to 14 kg.

0.05 - 7 µg/ml for UV and fluorescence detection, respectively. These methods have been evaluated and used to analyze plasma samples obtained from rats, guinea pigs, dogs, sheep and monkeys.

The drug also was administered to squirrel monkeys (2,3) guinea pigs and sheep (4) by the intrapulmonary route using an ultrasonic nebulizer or a Freon-based metered-dose inhaler. The plasma drug concentration following intrapulmonary administration provided evidence for either systemic or local absorption from the respiratory system.

To detect nanograms of drug in plasma resulting from aerosol administration, enhanced fluorescence sensitivity was obtained using the second set of chromatographic conditions described earlier (see Chromatographic Separation) which included a neutral mobile phase. Purging the mobile phase with nitrogen

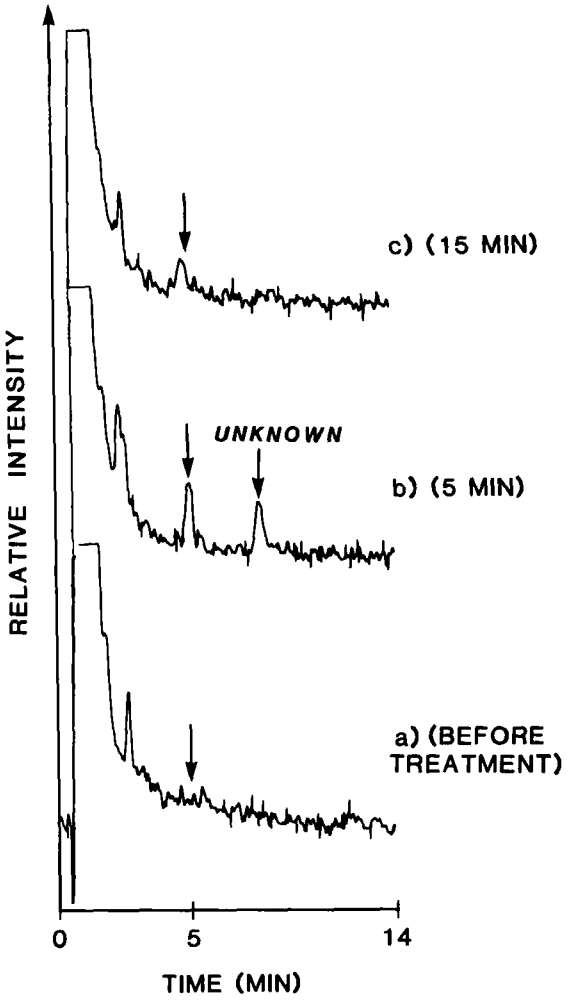


FIGURE 7 Chromatograms of guinea pig plasma after a nebulized drug solution was administered. See text for the chromatographic system. The injection volume was 50 µl. The traces shown are from plasma samples obtained from animals: a) before treatment, b) 5 min after treatment (1.2 ng/ml drug found in plasma), c) 15 min after treatment (0.6 ng/ml drug found in plasma). The detection limit in the guinea pig is lower (about 1 ng/ml) than any other species studied. The arrow indicates the location of the L-660,711 peak.

removed oxygen, a well-known fluorescence quencher. With this optimized system, detection was limited only by resolution of minor plasma components eluting near the drug peak of interest. This is illustrated in Fig 7 showing chromatograms obtained from plasma samples of the guinea pig before treatment, 5 minutes after and 15 minutes after administration of L-660,711. The detection limit obtained for normal plasma and serum samples from guinea pigs, sheep or monkeys was 1-5 ng/ml with a 25 µl injection volume. The calibration curve obtained for a range of concentration 5-300 ng/ml was linear with a correlation coefficient of 0.99. Over the concentration range 5-20 ng/ml the correlation coefficient was 0.95.

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