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SIMULTANEOUS ANALYSIS OF ANTIPYRINE AND LORAZEPAM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A novel analytical methodology was developed for simultaneous high-performance liquid chromatographic quantitation of antipyrine and lorazepam in biological fluids using phenacetin and flunitrazepam as internal standards, respectively. A gradient solvent system was used to achieve specificity and sensitivity for each of the compounds in biological fluids. The between-run coefficients of variation for replicate analysis of antipyrine and lorazepam in identical plasma samples were 9.4 and 6.4% at 60 μg/ml and 5.5 μg/ml, respectively, for antipyrine, and 9 1 and 9.9% at 110 ng/ml and 10.5 ng/ml, respectively, for lorazepam. Accuracy ranged from 99.7 to 101.4% at concentrations of 8, 45 and 105 μg/ml for antipyrine and from 94.8 to 95.8% at concentrations of 12, 55 and 125 ng/ml for lorazepam. This method requires only one organic extraction procedure for all four compounds, allows quantitation of these two drugs in samples also containing indocyanine green, and sample throughput following extraction is totally automated.

INTRODUCTION

Many drugs are metabolized and/or excreted by the liver, necessitating the need to adjust the dosage of some drugs in patients with hepatic dysfunction. However, multiple processes are involved in hepatic drug clearance, including liver blood flow, microsomal metabolism and synthetic reactions to form soluble conjugates (Fig. 1). This precludes the use of a single biochemical measurement to assess liver function and hepatic drug clearance in individual patients. For this reason, selected "model drugs" have been used to assess the efficiency of selected hepatic drug removal processes. Antipyrine is a drug commonly used to assess Phase I reactions since it undergoes hepatic oxidative metabolism by microsomal enzymes [1]. Lorazepam is a benzodiazepine which undergoes one-step conjugation with glucuronic acid by micro-

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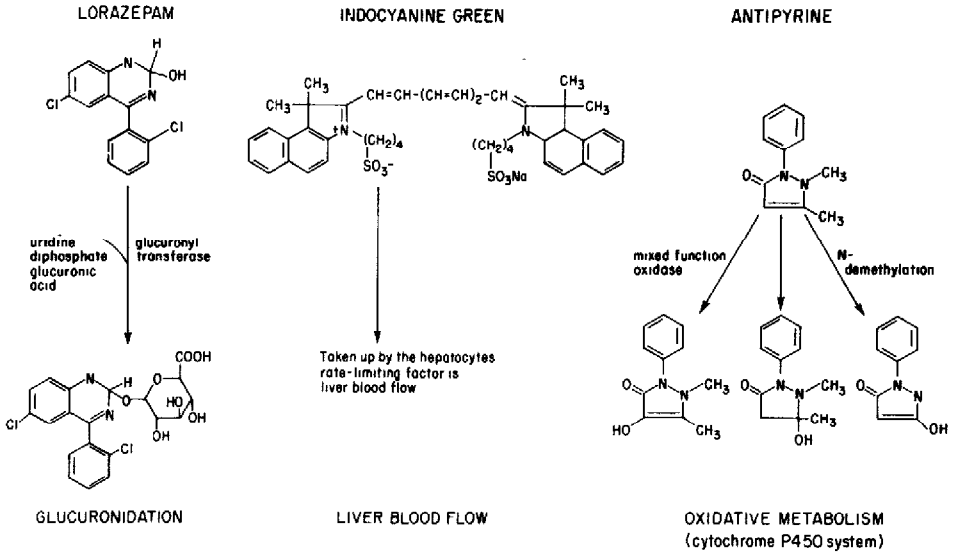

Fig 1 Scheme of pathways involved in hepatic drug clearance of three model substrates, lorazepam, indocyanine green and antipyrine.

somal glucuronyl transferases [2], and is therefore useful to assess Phase II reactions. Finally, indocyanine green (ICG) is a tricarbocyanine dye which is rapidly and efficiently removed from blood by hepatocytes and is not metabolized. At low doses (e.g., 0.5 mg/kg), the rate-limiting step for hepatic clearance of ICG is liver blood flow, making it a useful agent for assessing this component of hepatic drug clearance [3].

To yield a more complete assessment of liver function, relative to hepatic drug clearance, we simultaneously administer lorazepam (L), ICG (I) and antipyrine (A), and collect one set of blood samples from which the clearance of all three agents (LIA) is calculated. ICG can be measured by a simple spectrophotometric method [4]. This paper describes the high-performance liquid chromatographic (HPLC) method we developed to simultaneously quantitate lorazepam and antipyrine in plasma samples containing these two drugs and ICG. This eliminates the need for two extraction procedures and two separate HPLC assays, thereby decreasing analysis time and reducing the volume of blood required for analysis of these model drugs.

EXPERIMENTAL

Chemicals

Antipyrine was obtained from Aldrich (Milwaukee, WI, U.S.A.), acetophenetidine (phenacetin) from Sigma (St. Louis, MO, U.S.A.), lorazepam from Wyeth Labs. (Philadelphia, PA, U.S.A.), and flunitrazepam from Hoffman-La Roche (Nutley, NJ, U.S.A.). Distilled water was purified by a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.). HPLC-grade acetonitrile was used as purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Certified monobasic sodium phosphate, phosphoric acid (85%), sodium hydroxide, and reagent-grade anhydrous diethyl ether were used as received from Fisher Scientific (Springfield, NJ, U.S.A.).

High-performance liquid chromatography

The chromatographic system consisted of two Model 114M pumps, a Model 165 variable-wavelength dual-channel ultraviolet—visible detector, and a Model 450 data system/controller by Beckman Instruments (Berkeley, CA, U.S.A.). The system was interfaced with a Model SP8780XR autosampler by Spectra-Physics (San Jose, CA, U.S.A.). It was fitted with a 200-μl volumetric sample loop and Rheodyne Model 7010 injector. The analytical column used for separations was either a 10-μm, 30 cm × 3.9 mm I.D. μBondapak Phenyl, Waters Assoc. (Milford, MA, U.S.A.) or a comparable Phenomenex (Rancho Palos Verdes, CA, U.S.A.) 10-μm, 30 cm × 3.9 mm I.D. Bondex column. The column temperature was controlled by a Model LC-22A column heater by Bioanalytical Systems (West Lafayette, IN, U.S.A.).

A 2.3 cm × 3.9 mm I.D. guard column (Waters Assoc.), dry packed with μBondapak Phenyl 37—50 μm packing, was attached proximal to the analytical column (Waters Assoc.). Microvials (100 μl) for the autosampler, were purchased from Varian (Palo Alto, CA, U.S.A.) or Supelco (Houston, TX, U.S.A.). Microvial caps and septum were purchased from Spectra-Physics.

Separation of the four compounds of interest and interfering plasma components was achieved with a gradient solvent system. The mobile phase consisted of solvent A, which was acetonitrile—0.1% sodium phosphate buffer pH 3 (5:95), and solvent B, which was acetonitrile—0.1% sodium phosphate buffer pH 3 (70:30). The initial conditions for the first 2.5 min of the run were 80% A and 20% B. The solvent composition changed linearly over a period of 20 min to 45% A and 55% B. At 20 min, the solvent composition then changed linearly over a period of 3 min to 25% A and 75% B. At 25 min, the solvent composition changed over a period of 3 min back to initial conditions of 80% A and 20% B and equilibrated for 7 min. Therefore, the assay time was 35 min. The solvent flow-rate for the assay was 2 ml/min, and the column temperature was maintained at 40°C. Operating pressures under these conditions were about 100 bar.

Antipyrine was detected on one channel of the UV absorbance detector at 254 nm and 0.1 a.u.f.s. Lorazepam was detected on a separate channel of the detector at 229 nm and 0.005 a.u.f.s.

Integration of peak heights, peak-height ratios, calculation of standard curves, and computations of concentrations of antipyrine and lorazepam in patient samples were performed by the Beckman 450 data system.

Sample preparation

The compounds of interest and the internal standards were extracted from plasma as follows. An aliquot of patient's plasma (0.5 ml), 0.5 ml of water and 0.5 ml of 0.25 *M* sodium hydroxide were added to a 150 × 16 mm glass test tube, vortexed and incubated at room temperature for 20 min. Following incubation, 20 μl of an internal standard mixture (100 μg/ml phenacetin and 3000 ng/ml flunitrazepam) were added. The mixture was vortexed immediately. Diethyl ether (5 ml) was added immediately and the mixture was vortexed for 30 s. Since the stability of flunitrazepam is pH-dependent, it is important to quickly extract it into the ether phase from the strongly alkaline aqueous phase. Samples were then centrifuged at 900 *g* for 5 min and frozen in an

acetone dry ice bath for 5 min. The supernatant (diethyl ether) of the frozen mixture was decanted into 75 × 12 mm glass test tubes to be dried under nitrogen. The dried samples were reconstituted in 115 μl of acetonitrile—0.1% sodium phosphate buffer pH 3.0 (30:70). All of this solution was transferred (using the same pipet so as not to loose sample volume) to conical microvials for injection onto the HPLC system by the autosampler. To determine between-run precision of the assay, five to ten samples of two known concentrations of each compound were assayed each day, on three different days. Ten determinations of two known concentrations of each compound assayed within a day determined within-run precision. Accuracy was determined by assaying plasma to which a known amount of drug had been added. These samples were prepared at three different concentrations by one technologist and assayed in triplicate by a second technologist unaware of the target values.

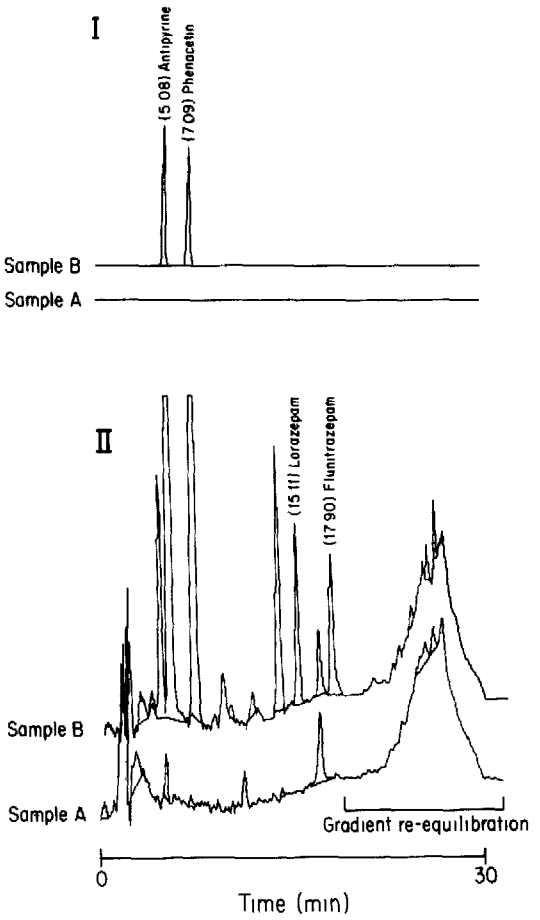

Fig. 2. Chromatograms of a patient's plasma obtained prior to administration of LIA (sample A) and plasma obtained 1 min after an LIA dose (sample B). Panel I depicts the chromatograms of column eluate monitored at 254 nm (0.1 a.u.f.s.) for quantitation of antipyrine. Panel II, simultaneously monitored at 229 nm (0.005 a.u.f.s), is utilized for quantitation of lorazepam.

RESULTS

With this newly developed HPLC assay, all compounds of interest are well resolved from each other and potentially interfering UV-absorbing compounds in plasma (Fig. 2). Lorazepam was monitored at 229 nm, to achieve maximum sensitivity. Antipyrine was quantitated from chromatograms monitored at 254 nm, because sensitivity was adequate and there is a potentially interfering peak at 229 nm. Retention times and capacity factors of the four compounds of interest are summarized in Table I. Drugs tested and found not to interfere with the assay included acetaminophen, trimethoprim, sulfamethoxazole, allopurinol and ICG.

There was a linear relationship between peak-height ratio and concentration for both antipyrine (6.25—100 μg/ml) and lorazepam (12.5—200 ng/ml)

TABLE I

SEPARATION OF ANTIPYRINE, PHENACETIN, LORAZEPAM AND FLUNITRAZEPAM BY REVERSED-PHASE HPLC

Compound	Retention time (min)	Capacity factor (k')
Antipyrine	5.08	1.70
Phenacetin	7.09	2.77
Lorazepam	15.11	7.04
Flunitrazepam	17.90	8.52

TABLE II

WITHIN-RUN PRECISION OF ANTIPYRINE AND LORAZEPAM

$n = 10$.

Compound	Actual concentration	Measured concentration (mean ± S.D.)	Accuracy (%)	C.V. (%)
Antipyrine	60	65.9 ± 2.6	109.9	4.0
(μg/ml)	5.5	5.7 ± 0.3	103.1	4.5
Lorazepam	110	110.5 ± 7.3	100.4	6.6
(ng/ml)	10.5	9.77 ± 1.0	93.0	10.7

TABLE III

BETWEEN-RUN PRECISION OF ANTIPYRINE AND LORAZEPAM

$n = 20$.

Compound	Actual concentration	Measured concentration (mean ± S.D.)	Accuracy (%)	C.V. (%)
Antipyrine	60	61.1 ± 5.8	101.8	9.4
(μg/ml)	5.5	5.5 ± 0.4	100.5	6 4
Lorazepam	110	111.2 ± 10.1	101.1	9.1
(ng/ml)	10.5	9 8 ± 1.0	93.0	9.9

TABLE IV

ACCURACY OF ANTIPYRINE AND LORAZEPAM

$n = 3$.

Compound	Actual concentration	Measured concentration (mean ± S.D.)	Accuracy (%)
Antipyrine (μg/ml)	8	8.08 ± 0.4	101.0
	45	44.9 ± 4.1	99.7
	105	106.5 ± 5.2	101.4
Lorazepam (ng/ml)	12	11.4 ± 0.3	94.8
	55	52.5 ± 2.4	95.4
	125	119.7 ± 4.7	95.8

over the concentration ranges evaluated ($r^2 > 0.98$) permitting quantitation by a single-point calibration method using the Beckman 450 integrator. As summarized in Tables II—IV this method is both precise and accurate. Between-run coefficients of variation (C.V.) were 9.4% at 60 μg/ml and 6.4% at 5.5 μg/ml for antipyrine and 9.1% at 110 ng/ml and 9.9% at 10.5 ng/ml for lorazepam. Within-run C.V. was 4.0% at 60 μg/ml and 4.5% at 5.5 μg/ml for antipyrine and 6.6% at 110 ng/ml and 10.7% at 10.5 ng/ml for lorazepam. Accuracy, defined as a percentage of the target value, was 99.7 to 101.4% (mean 100.7%, S.D., 0.89%) for antipyrine, at concentrations ranging from 8 to 105 μg/ml. Accuracy was 94.8 to 95.8% (mean 95.3%, S.D. 0.50%) for lorazepam at concentrations ranging from 12 to 125 ng/ml (Table IV).

Fig. 3 depicts the plasma concentration—time curves for the three model substrates (LIA) in a representative patient, measured by the methods described herein. The dosage of LIA for these studies is 0.03 mg/kg lorazepam (maximum 2 mg), 0.5 mg/kg ICG, and 10 mg/kg antipyrine.

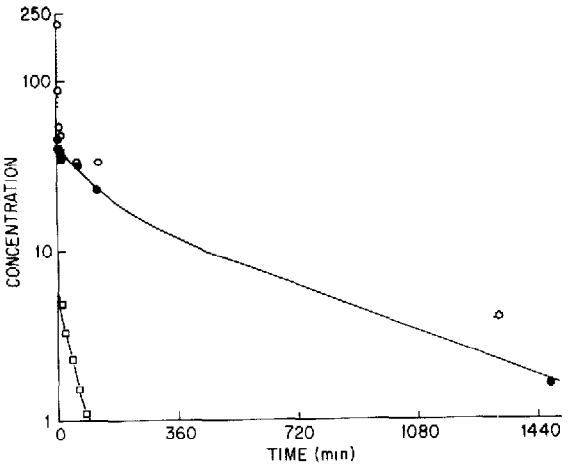

Fig. 3. Semilogarithmic plot of lorazepam (○), ICG (□) and antipyrine (●) plasma concentrations versus time following administration of LIA to a patient. Lorazepam concentrations are in ng/ml, antipyrine and ICG are in μg/ml.

DISCUSSION

The assay described in this paper permits simultaneous analysis of antipyrine and lorazepam in plasma, permitting one to assess multiple processes involved in hepatic drug clearance by simultaneously giving a mixture of these model drugs. This assay is selective and sensitive enough to allow quantitation of antipyrine and lorazepam from a single plasma sample of relatively low volume (i.e. 500 μl). The new method has overcome limitations which led others to conclude that "HPLC probably will not become applicable to pharmacokinetic studies of lorazepam because of its very low plasma concentrations" [5]. Moreover, simultaneous quantitation of these two drugs in small-volume plasma samples has not previously been described, preventing utilization of multiple markers of liver function in pediatric populations. The assay described herein not only makes it possible to assay these two model substrates in small volumes of plasma, but also makes such studies more feasible since only one HPLC analysis is required.

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