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QUANTITATIVE DETERMINATION OF TOLAZOLINE IN SERUM AND URINE

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SUMMARY

A high-performance liquid chromatographic procedure is described for the analysis of tolazoline in serum and urine. This assay procedure is suitable for the analysis of micro-samples (50 or 100 μl serum and 100 μl urine). Samples are extracted in a single step and injected into a reversed-phase high-performance liquid chromatography system for detection at 210 nm. The clinical applicability of this assay is demonstrated by the determination of tolazoline serum and urine concentrations in neonates. In addition, the presence of urine conjugates and the extent of serum protein binding were investigated. This assay procedure has the required sensitivity (0.1 μg/ml), accuracy and precision for both routine monitoring and pharmacokinetic characterization of tolazoline in neonates and adults.

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INTRODUCTION

Tolazoline [4,5-dihydro-2-(phenylmethyl)-1H-imidazole] has been used as a vasodilator in the treatment of several peripheral vascular disorders in adults since the 1940s [1]. The pharmacology of this agent is complex, with studies demonstrating histaminergic, α-adrenergic blocking, sympathomimetic, parasympathomimetic and direct vasodilator activities [2, 3]. Several recent investigations have reported the use of tolazoline for the treatment of neonatal pulmonary hypertension [4, 5]. While tolazoline has been reported to be successful in the treatment of this disorder, a high incidence of adverse effects has been associated with its use in neonates [5, 6]. This narrow range between therapeutic and toxic doses and the paucity of information available on the pharmacokinetics of tolazoline in neonates [7] indicate the need to be able to monitor serum concentrations in these patients. Further, since the drug appears to be primarily excreted by the kidney [8], the determination of both serum and urine concentrations of tolazoline is required to accurately characterize its pharmacokinetics in neonates.

Several methods for the determination of tolazoline have previously been reported [8—11]. These methods were not suitable for the analysis of tolazoline in neonates because of the lack of specificity and sensitivity, and large sample volume requirements (> 200 μl). One previously published method has the desired sensitivity and sample volume size, but requires the use of a mass spectrometer and an extensive sample workup procedure involving extraction, freezing at 0°C and derivatization [12]. In addition, none of the previously published assay procedures has been utilized for the analysis of both serum and urine.

We now report a high-performance liquid chromatographic (HPLC) assay for the quantitation of tolazoline in serum and urine. This assay is a rapid, one-step extraction procedure and is suitable for the analysis of microsamples (50--100 μl). The clinical applicability of this method is shown by the analysis of tolazoline serum and urine samples from neonatal patients.

MATERIALS AND METHODS

Standards and reagents

Tolazoline hydrochloride was furnished by Ciba Pharmaceutical (Summit, NJ, U.S.A.). Naphazoline hydrochloride (internal standard), monobasic potassium phosphate (KH_2PO_4) and β-glucuronidase (EC 3.2.1.31) were obtained from Sigma (St. Louis, MO, U.S.A.). ACS reagent grade potassium bicarbonate ($KHCO_3$), potassium carbonate (K_2CO_3), sodium acetate, acetic acid and HPLC grade solvents (acetonitrile and methylene chloride) were obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.). HPLC quality water was obtained through a Millipore Milli-Q^{TM} water purification system (Bedford, MA, U.S.A.) which was fed with distilled water.

The mobile phase was composed of phosphate buffer, 0.02 *M* KH_2PO_4, pH 3.75—acetonitrile (3:2, v/v). The phosphate buffer was filtered through a 0.45-μm Millipore HA filter and then passed through a Waters μBondapak® C_{18} column to remove organic compounds which had produced an unstable

chromatographic baseline. The buffer pH was adjusted to 3.75 with 85% phosphoric acid. The phosphate buffer and acetonitrile were then mixed and filtered through a Nuclepore polyester 0.4-μm membrane filter (Pleasanton, CA, U.S.A.).

Extraction procedure

The extraction of tolazoline and internal standard was performed in a single procedure. A serum or urine sample (50 or 100 μl) was placed into a 15-ml conical tube along with 30 μl of the internal standard aqueous solution (1.0 μg/ml for serum and 100 μg/ml for urine), 250 μl of extraction buffer (0.1 *M* $KHCO_3$, 0.1 *M* K_2CO_3, pH 10.0) and 5.0 ml of methylene chloride. The contents were sealed with a PTFE-lined cap, shaken mechanically for 20 min and centrifuged for 5 min at 1500 *g*. The aqueous layer at the top was removed by aspiration. The organic layer was transferred to a clean 75 × 100 mm tube and evaporated to dryness under a gentle stream of nitrogen in a water bath at 35°C. The dry residue was reconstituted with 100 μl (500 μl for urine samples) of 0.02 *M* KH_2PO_4, pH 3.75, and vortexed for 15 sec. The reconstituted serum extract was transferred to a WISP™ limited volume insert sample vial and 90 μl were injected into the chromatograph. For urine samples, the reconstituted extract was transferred into a limited volume glass insert (Sun Brokers, Wilmington, NC, U.S.A.) and 10 μl were injected into the chromatograph.

Liquid chromatographic conditions

A Waters Assoc. Model 6000A chromatography pump (Milford, MA, U.S.A.) was used to control the flow of the mobile phase at 1.2 ml/min. Samples were injected into the system using a WISP Model 710B sample processor (Waters Assoc.). A Whatman® guard column (5 cm × 2 mm I.D.) filled with Co:Pell ODS (Clifton, NJ, U.S.A.) was placed in series with the analytical column, a Waters Resolve® column, 5-μm Spherical C_{18}. The detection of the eluted peaks was accomplished by a Kratos Spectro-Flow 773 variable-wavelength detector (Westwood, NJ, U.S.A.) operated at a wavelength of 210 nm. The retention times for tolazoline and naphazoline were 7 and 10 min, respectively. The detector signal was recorded and peak area quantitated with a Hewlett-Packard 3390A integrator (Avondale, PA, U.S.A.).

Hydrolysis of urine conjugates

Since our preliminary pharmacokinetic studies in neonates found only about 80% of the dose excreted unchanged in the urine [13], three methods known to hydrolyze urine conjugates were performed to determine if conjugation represented another route for tolazoline elimination. For each hydrolysis method, three sets of samples were analyzed. The first set was drug-free urine to determine whether the hydrolysis procedure resulted in any compounds which would interfere with the assay. The second set consisted of drug-free urine spiked with known amounts of tolazoline to confirm the stability of tolazoline during the hydrolysis procedure. The third set was urine samples from patients receiving tolazoline. All samples were analyzed for tolazoline, using the above HPLC procedure, both before and after the hydrolysis procedure.

Acid hydrolysis was performed by adding 0.5 ml of concentrated hydrochloric acid to a 0.5-ml aliquot of urine. The samples were placed in PTFE-sealed glass tubes in boiling water for 1 or 6 h. A third set of samples was allowed to stand at room temperature for 1 h. After removal from the water bath, the samples were allowed to cool and then neutralized by the addition of 0.5 ml of 12 *M* sodium hydroxide. An aliquot (100 μl) was removed and extracted according to the procedure outlined above.

Basic hydrolysis was performed by adding 0.5 ml of 12 *M* sodium hydroxide to a 0.5-ml aliquot of urine. The samples were placed in PTFE-sealed glass tubes in boiling water for 1 or 6 h. A third set of samples was allowed to stand at room temperature for 1 h. After cooling, an aliquot (100 μl) was extracted according to the procedure outlined above.

Enzyme hydrolysis was performed by adding 0.5 ml of acetate buffer and 100 μl of β-glucuronidase (approx. 1000 U/ml) to a 0.5-ml aliquot of urine. The samples were incubated at 37°C for 24 h. An aliquot (100 μl) of the hydrolyzed sample was then extracted using the procedure outlined above.

Equilibrium dialysis procedure

The extent of tolazoline binding to serum proteins was examined by equilibrium dialysis. A Spectrum equilibrium dialysis system was used with 1.0-ml PTFE cells and Spectra/Por®-2 membranes (Spectrum Medical Industries, Los Angeles, CA, U.S.A.). Serum, 0.7 ml, was introduced into one compartment of the cell and dialyzed against 0.7 ml of 0.01 *M* phosphate buffer (pH 7.4) made isotonic with sodium chloride. During dialysis the cells were placed in a water bath at 37°C and rotated at 10 rpm. At the end of the dialysis procedure, the concentration of tolazoline in both compartments was assayed by the HPLC procedure described above. The time to reach equilibrium was determined by dialyzing blank adult serum spiked with a known amount of tolazoline for 0, 30, 45, 60, 90, 120 and 240 min. When the time to reach equilibrium had been established, replicate samples of adult (n = 3) and neonatal (n = 2) serum spiked with tolazoline were dialyzed on three different occasions to determine the extent of serum protein binding.

RESULTS AND DISCUSSION

The serum standard curve was linear over the range of tolazoline concentrations from 0.1 to 15.0 μg/ml. The standard curve was found to be stable over a seven-week period with a coefficient of variation for the slope of 4.2% for nine curves run over this time period. Pooled adult serum was used for the standard curve and control samples because of the lack of availability of large amounts of blank neonatal serum. No difference was observed in chromatograms obtained from adult and neonatal serum samples (Fig. 1).

In order to improve the sensitivity and allow for a smaller sample size as compared to the previously published HPLC procedure [11], a new extraction procedure was developed using a new internal standard. The new procedure resulted in an extraction efficiency of 95.6 ± 2.3% S.D. at a concentration of 1.0 μg/ml. The minimum detectable concentration was 0.1 μg/ml with a coefficient of variation for replicate samples (n = 5) of 10.3%. No interference

from endogenous compounds in the serum was observed (Fig. 1). The assay procedure was also examined for possible interference from hemolyzed serum or other drugs (Table I). Serum samples spiked with these drugs and a blank hemolyzed neonatal serum sample were extracted by the same procedure as the tolazoline serum samples. No peaks which would interfere with tolazoline or internal standard were observed.

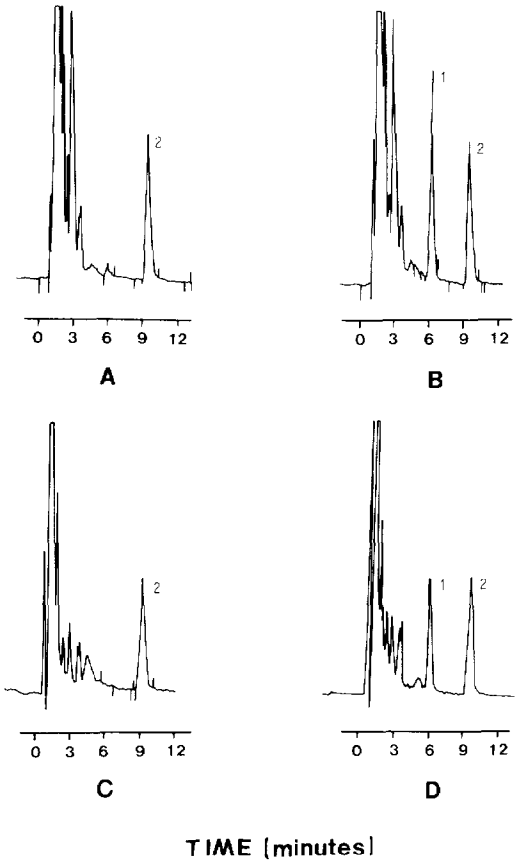

Fig. 1. Chromatograms comparing adult and neonate serum extracts. (A) Neonate blank with internal standard; (B) neonate patient with a measured tolazoline serum concentration of 0.76 μg/ml; (C) blank adult serum with internal standard; (D) adult serum spiked with 0.39 μg/ml tolazoline. Peaks: 1 = tolazoline; 2 = naphazoline, internal standard.

TABLE I

DRUGS TESTED FOR INTERFERENCE IN THE ASSAY PROCEDURE

Carbamazepine	Digoxin	Acetaminophen
Ethosuximide	Disopyramide	Chloramphenicol
Phenobarbital	Lidocaine	Desipramine
Phenytoin	Methotrexate	Dopamine
Primidone	Procainamide	Gentamicin
Valproic acid	N-Acetyl procainamide	Imipramine
	Propranolol	Salicylates
	Quinidine	
	Theophylline	

The accuracy and precision data for the serum assay based on five determinations at five different concentrations are summarized in Table II. Neonatal patients receiving the standard dosage regimens were found to have tolazoline serum concentrations ranging from 2.0 to 13.8 μg/ml. The intra-day and inter-day coefficients of variation in this concentration range were less than 5% and 8%, respectively.

The standard curve for the urine assay was linear over the concentration range 0.03—2.0 mg/ml and was stable over a ten-week period with a coefficient of variation for the slope of 3.9% for nine curves run over this time period. The accuracy and precision data for the urine assay based on four determinations at four different concentrations are summarized in Table III. Adult, drug-free urine was used to prepare standard curve and control samples. No difference was observed in chromatograms obtained from adult and neonatal urine samples (Fig. 2). Urine samples obtained from neonates receiving tolazoline had urine concentrations ranging from 0.03 to 0.8 mg/ml.

The results of the three urine hydrolysis procedures are summarized in Table IV. No evidence for the presence of urine conjugates of tolazoline could be found from any of the three procedures. The three urine samples spiked with a known amount of tolazoline had a slight decrease in tolazoline concentration after 1 and 6 h of acid hydrolysis owing to a slow breakdown of tolazoline in the acid medium. A similar decrease in tolazoline concentration was observed in the two neonatal patient samples. Following basic hydrolysis for 1 and 6 h, both spiked and patient samples had extensive breakdown of

TABLE II

SERUM TOLAZOLINE ASSAY PRECISION AND ACCURACY

Theoretical control concentration (μg/ml)	Mean measured concentration (μg/ml)	Intra-day coefficient of variation (%)	Inter-day coefficient of variation (%)
0.25	0.27	9.6	10.0
0.74	0.73	6.6	6.1
1.99	2.00	4.2	7.7
3.14	2.74	3.6	5.5
12.04	11.93	2.0	1.3

TABLE III

URINE TOLAZOLINE ASSAY PRECISION AND ACCURACY

Theoretical control concentration (mg/ml)	Mean measured concentration (mg/ml)	Intra-day coefficient of variation (%)	Inter-day coefficient of variation (%)
0.055	0.055	9.3	8.2
0.45	0.44	2.1	7.0
0.90	0.87	7.3	10.0
1.80	1.84	4.9	7.0

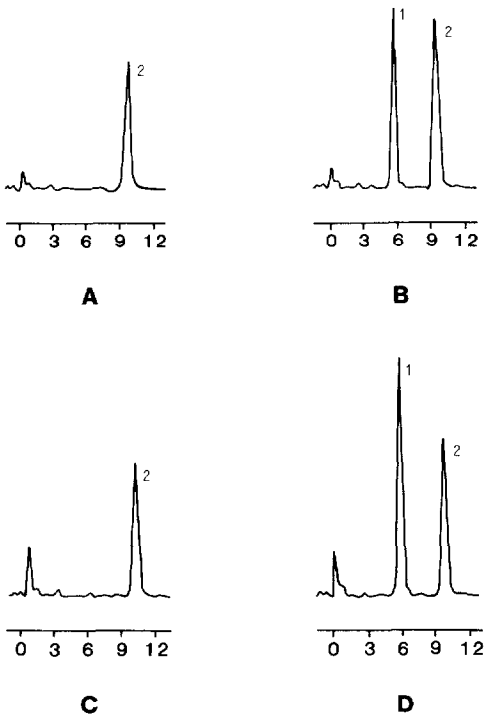

Fig. 2. Chromatograms comparing adult and neonate urine extracts. (A) Neonate blank with internal standard; (B) neonate patient with a measured tolazoline urine concentration of 0.09 mg/ml; (C) blank adult urine with internal standard; (D) adult urine spiked with 0.12 mg/ml tolazoline. Peaks: 1 = tolazoline, 2 = naphazoline, internal standard.

TABLE IV

URINE HYDROLYSIS

Results are expressed as percent of prehydrolysis tolazoline concentration.

Sample	Acid hydrolysis*			Basic hydrolysis*			Enzyme hydrolysis
	0 h**	1.0 h	6.0 h	0 h**	1.0 h	6.0 h	24 h
Spike No. 1	100.0	84.6	84.6	100.0	1.1	0	105.5
Spike No. 2	101.1	85.7	85.7	88.9	1.1	0	105.5
Spike No. 3	107.7	92.3	92.3	100.0	2.5	0	110.0
Patient No. 1	94.1	88.2	88.2	70.6	5.9	0	100.0
Patient No. 2	111.1	91.7	75.0	72.2	2.8	0	111.1

*Heated in a boiling water bath for time indicated.
**Allowed to stand at room temperature for 1 h.

tolazoline. The samples which were incubated in base at room temperature for 1 h showed only a minimal change in tolazoline concentration. Enzyme hydrolysis with β-glucuronidase produced no change in tolazoline concentration for either the spiked or neonatal patient samples. These results would

indicate that conjugation of tolazoline in neonates does not represent a major route of elimination.

The equilibrium dialysis procedure indicated minimal (< 10%) serum protein binding of tolazoline in either adults or neonates.. Equilibrium was achieved in 60 min. The mean recovery of tolazoline following the 60-min dialysis procedure was 100.4 ± 2.5% S.D. (n = 5). The intra-day and inter-day coefficient of variation for replicate samples (n = 3) with the dialysis procedure was 6.0 and 8.2%, respectively. The mean percent of tolazoline bound to serum proteins was 6.9% and 6.3% in adult and neonatal serum, respectively.

This assay procedure, therefore, has the sensitivity, accuracy and precision needed for both routine monitoring and pharmacokinetic characterization of tolazoline in neonates and adults. This method may be used to quantitate tolazoline in both serum and urine. While the procedure has been described here using 100-μl samples, the sensitivity of the assay procedure allows for a sample volume as small as 50 μl to be used when sample size is limited. This small sample size requirement and lack of interference from other drugs and hemolyzed samples make the procedure especially suitable for neonates where serum samples are difficult to obtain and sample volumes are limited. The simple and rapid extraction procedure allows the analysis of a large number of samples at one time. This method is currrently being used to study the pharmacodynamics and pharmacokinetics of tolazoline in neonates with pulmonary hypertension.

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