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Note

Simultaneous determination of doxepin and nordoxepin in serum using high-performance liquid chromatography

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Doxepin (DOX) is a tricyclic antidepressant which is widely used for the therapeutic management of endogenous depression and, more recently, for the treatment of peptic ulcer disease [1, 2]. The pharmacokinetics of DOX after oral administration are complicated by the extensive first-pass hepatic metabolism which decreases the extent of absorption (bioavailability). There is considerable inter-patient variability in the first-pass effect of DOX [3]. Furthermore, DOX undergoes oxidative demethylation in the liver to form nordoxepin (NDOX) which is pharmacologically active [4]. As a result the extent, and rate, of absorption of DOX from oral dosage forms influences the amount of NDOX that is formed during the first-pass effect. Thus, oral bioavailability is an important factor determining a patient's clinical response to DOX.

There is relatively little information on the oral bioavailability of DOX because of the lack of a specific and sensitive assay method for conveniently quantitating the relatively low serial serum DOX and NDOX concentrations, respectively, obtained after a single dose. High-performance liquid chromatography (HPLC) has become popular for quantitating serum antidepressant concentrations. Most HPLC analytical methods have been designed to measure simultaneously all or most of the tricyclic antidepressants, including DOX, and this has resulted in either extensive sample pretreatment or limited sensitivity [5–9]. Other HPLC assay methods measure DOX but not its active metabolite [10, 11]. Ziegler et al. [12] described an assay procedure for DOX and NDOX which was sensitive and

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specific, but not convenient for bioavailability trials because it utilized gas chromatography–mass fragmentography. Gas chromatographic methods, in general, also require derivatization of secondary amine antidepressants before assay to facilitate separation from parent amines. Radioimmunoassays and enzyme immunoassays are non-specific and lack the necessary sensitivity for single-dose bioavailability studies.

The objective of this research was to develop an HPLC method for DOX and NDOX that had the necessary sensitivity and specificity for use in single-dose bioavailability trials, yet, was convenient for the assay of the large number of serum samples that typically arise from these types of pharmacokinetic studies.

EXPERIMENTAL

Equipment

An HPLC system consisting of a dual-piston pump (Model 6000A, Waters Assoc., Milford, MA, U.S.A.) set to a flow-rate of 1.5 ml/min, a variable-wavelength UV detector (Model 783, Kratos Analytical, Ramsey, NJ, U.S.A.) set to 214 nm and a range of 0.001 a.u.f.s., an automatic sample injector (Model ISS-100, Perkin-Elmer, Norwalk, CT, U.S.A.) and a data module (Model 740, Waters Assoc., or Model 3390, Hewlett-Packard, Rockville, MD, U.S.A.) was used at ambient temperature for all analyses. The analytical column was a 150 mm × 4.6 mm stainless-steel column packed with Spherisorb 5-μm cyanopropyl-bonded silica gel (Phenomenex, Rancho Palos Verdes, CA, U.S.A.).

For solid-phase extraction, 1 ml cyanopropyl-bonded extraction columns (lot No. 133548) and a vacuum elution processing station (AI 6000) were obtained from Analytichem International (Harbor City, CA, U.S.A.). Polypropylene test tubes were used for all solutions and eluents containing DOX and NDOX.

Reagents

HPLC-grade methanol and phosphoric acid (85%, v/v) were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.); triethylamine (Fisher Scientific, Silver Spring, MD, U.S.A.) was also HPLC grade. All other reagents were analytical grade and were used as obtained including sodium *n*-heptane sulfonic acid (Eastman-Kodak, Rochester, NY, U.S.A.) and N-(*n*-octyl)diethylamine (Alfa Products, Danvers, MA, U.S.A.). Water was doubly distilled and deionized before use. For the chromatography, the mobile phase consisted of 55% methanol in 0.02 *M* phosphoric acid containing 0.05% (N-(*n*-octyl)dimethylamine; the pH of the mobile phase was approximately 2.4.

Standards

For use as analytical standards, doxepin hydrochloride powder (lot No. 8P039-02QCS, Pfizer Labs., New York, NY, U.S.A.) was obtained as a mixture containing 76.3% *E*-isomer and 12.6% *Z*-isomer, respectively. Nordoxepin powder (lot No. 4K032-02QCS, Pfizer Labs.) was obtained as the *E*-isomer (97.7%). Imipramine hydrochloride (IMI, lot No. G-1), the internal standard, was obtained from the United States Pharmacopeia (Rockville, MD, U.S.A.). Concentrations

of DOX, NDOX and IMI were calculated as free bases. Stock solutions containing 1.0 mg/ml DOX, 1.0 mg/ml NDOX and 1.0 mg/mol IMI were prepared in methanol. Stock solutions were labelled and stored at −20°C where they were stable for up to three months. A working stock solution containing 5 mg/l DOX and NDOX was prepared by diluting appropriate aliquots of the stock solutions with 20% serum in water and stored at 4°C.

Blank (drug-free) pooled serum was obtained from four normal healthy volunteers. Serum reference standards containing 2, 5, 10, 25, 50 and 100 ng/ml DOX and NDOX were prepared by diluting appropriate aliquots of the working stock solution with blank serum. Serum standards were stored at −20°C where they were stable for at least three months. The internal standard solution was prepared to contain 0.2 μg/ml IMI by diluting an appropriate volume of the working internal standard stock solution with 0.05 *M* sodium *n*-heptanesulfonic acid.

Procedure

The solid-phase extraction columns were activated by washing them with, in sequence, 1 ml of distilled water and 1 ml of methanol. Care was taken to avoid air reaching the column packing during the washing step. A 1-ml aliquot of the blank serum, the serum reference standard or the unknown serum specimen was prepared for extraction by adding 0.25 ml of internal standard solution. The entire sample was transferred to the top of the column and a vacuum was applied. The column was then washed with 1 ml of distilled water and 1 ml of methanol–water (50:50) and the eluent was discarded. The column was allowed to air dry. The column was then eluted with 1.0 ml of 0.8% triethylamine in methanol and the eluent was collected and evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was reconstituted with 0.25 ml of HPLC mobile phase and 50 μl were injected into the chromatograph.

Calculations

Peak-height ratios of DOX/IMI and NDOX/IMI for the serum standards were determined by numerical integration with the automatic data reduction system. A standard curve was prepared by plotting peak-height ratio as a function of concentration for each compound and determining the best-fit line by linear regression analysis.

Validation

Intra- and inter-day precision were determined from standard curves prepared from serum standards containing 2–100 ng/ml and assayed on three to seven different occasions. Accuracy was determined by comparing the target (spiked) concentration in the serum standards to the concentrations determined from the best-fit standard curve line. Sensitivity was defined as the lowest concentration that produced a signal-to-noise ratio of 3:1. Extraction efficiency was determined by comparing the absolute peak height of doxepin and nordoxepin obtained from extracted samples to the peak height obtained by direct injection of a methanol standard containing a known amount of each compound.

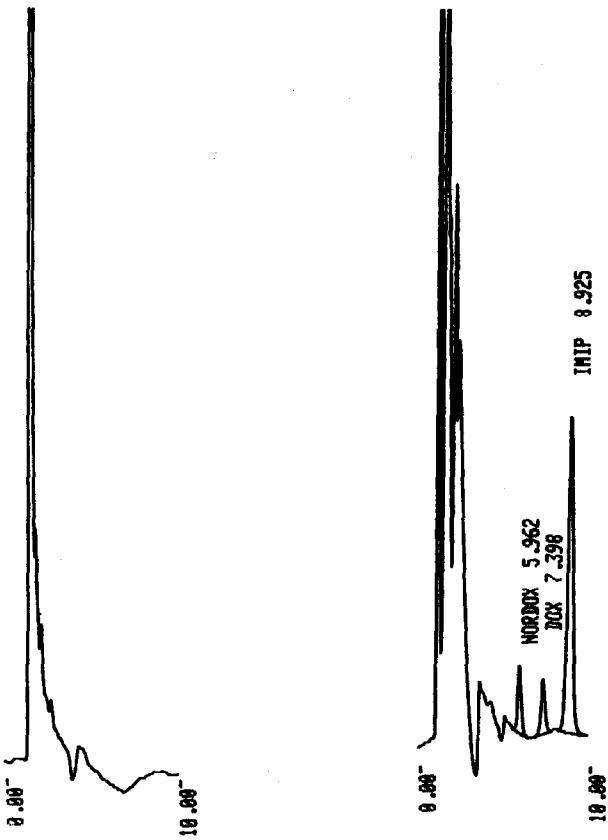

Fig. 1. Typical chromatograms of blank human serum (left) and serum standard containing 10 ng/ml doxepin (DOX), 10 ng/ml nordoxepin (NORDOX) and internal standard (imipramine, IMIP) (right).

RESULTS

Fig. 1 shows a typical chromatogram of blank serum and serum containing 10 ng/ml DOX and NDOX, which were assayed according to the described procedure. The extract from the blank serum showed a clean baseline showing that endogenous substances did not interfere with DOX and NDOX quantitation. The retention times of DOX, NDOX and IMI were 6, 7.5 and 9 min, respectively, and baseline separation of each component was easily achieved. The peak shapes were symmetrical with no evidence of tailing.

Standard curves of DOX and NDOX were linear up to 100 ng/ml. Standard curves for DOX had a mean ($n=3$) slope of 0.017 ± 0.0002 and standard curves for NDOX had a mean ($n=3$) slope of 0.020 ± 0.0002; for each individual standard curve the intercepts were not significantly different from zero and the correlation coefficients were > 0.99. The intra- and inter-day precision data for the assay are shown in Table I. The intra-day precision of the DOX assay ranged from $\pm 14\%$ at 2 ng/ml ($n=6$) to $\pm 1.5\%$ at 100 ng/ml ($n=7$). The inter-day

TABLE I

INTRA-DAY AND INTER-DAY PRECISION DATA FOR ASSAY OF DOX AND NDOX

Values are mean (±relative standard deviation).

Concentration added (ng/ml)	Concentration found (mean ± %R.S.D.) (ng/ml)		n
	DOX	NDOX	
Intra-day			
100	100.0 ± 1.5	100.0 ± 1.7	7
50	51.9 ± 2.7	51.9 ± 3.1	3
25	24.3 ± 4.1	24.6 ± 1.0	7
10	9.6 ± 3.5	9.9 ± 8.4	3
5	4.8 ± 10.5	4.8 ± 6.2	7
2	1.7 ± 13.8	1.8 ± 10.7	6
Inter-day			
100	99.0 ± 0.9	98.9 ± 0.8	7
50	52.5 ± 3.0	52.7 ± 3.3	3
25	24.3 ± 3.6	24.5 ± 0.2	7
10	9.6 ± 6.8	9.9 ± 8.6	3
5	5.0 ± 14.8	4.4 ± 5.3	7
2	1.7 ± 9.5	1.6 ± 7.5	6

precision for DOX was ±9.5% at 2 ng/ml and ±0.9% at 100 ng/ml. Over the same concentration range the intra- and inter-day precision of the NDOX assay was ±0.8 to 11%. The accuracy calculated as 100×[(target concentration − calculated concentration)/target concentration] was approximately ±16% at 21 ng/ml and ±1% at 100 ng/ml for both DOX and NDOX. The sensitivity of the assay was 1 ng/ml for DOX and NDOX, and the mean extraction efficiency was >90% for both compounds over the range of 2–100 ng/ml.

DISCUSSION

The pharmacokinetics and bioavailability of DOX are characterized by large inter-individual variability related primarily to differences in first-pass metabolism and subsequent hepatic clearance. Although the absolute bioavailability of DOX is unknown, because there is no reference intravenous dosage form, the relative bioavailability of oral DOX dosage forms can be easily determined using a suitable assay procedure. Because DOX is converted to an active metabolite (NDOX) on the first pass, it is important for an assay procedure to simultaneously measure both compounds. Relative bioavailability is very important clinically in the selection of a DOX dosage form for patients.

The mean peak serum DOX concentration observed in ten normal volunteers was reported to be 43 ng/ml following a single 100-mg oral dose of DOX [13]. The corresponding peak NDOX serum concentration was reported to be between 10 and 20 ng/ml. Serum DOX concentrations declined in a log linear fashion and reached 1–2 ng/ml at 100 h post-dose while the NDOX concentrations had declined

to about 5 ng/ml. Therefore, to study the single-dose oral bioavailability of DOX an assay procedure needs to be simple, specific and sensitive to at least 2 ng/ml and its must be able to quantitate both DOX and NDOX. The assay in this report meets these specifications.

The use of solid-phase extraction in this assay has obviated the traditional need for sample alkalinization, extraction into organic solvents and, in some cases, back-extraction into acid. In the past these complex manipulations have resulted in lower recoveries, adsorption loss of drug and generally unsatisfactory precision at low concentrations. The elution solvent was designed to maximize the recovery of DOX and NDOX. The recovery of DOX and NDOX in this study was virtually complete and we did not observe any loss of drug due to adsorption to the disposable polypropylene tubes used in the assay. The relatively quick extraction step also reduced technician time, decreased sample analysis turn-around-time and allowed for high recoveries from low sample volumes.

HPLC in the reversed-phase mode has been the method of choice for the measurement of serum antidepressant drug concentrations because of the compatibility of the chromatographic system with the biological matrix. In addition reversed-phase columns offer long-term stability, rapid equilibration with mobile phase and usually good assay reproducibility. In this assay we opted for a cyanopropyl-bonded column because it eliminated the peak tailing and longer retention times observed on C_{18} columns. As shown in Fig. 1 the assay run time is only 9 min which facilitates the serial analyses of large numbers of serum specimens obtained from bioavailability studies.

Since it was well known that tertiary amines, like DOX, did not chromatograph as well as ionized compounds, we opted to use an ion-pair reagent in the eluent. Because the pK_a values of DOX and NDOX are approximately 9.5–10, the eluent pH was reduced to 2.4 using phosphoric acid to assure complete ionization of DOX and NDOX. Separation was achieved as an ion pair using N-(*n*-octyl)dimethylamine, which also helped reduce the retention times of DOX and NDOX by decreasing adsorption to the column packing. Both phosphoric acid and N-(*n*-octyl)dimethylamine were transparent at 214 nm, the wavelength of maximum absorbance for DOX and NDOX. A concentration of 55% methanol in the mobile phase assured baseline separation of DOX and NDOX and good peak symmetry; concentrations less than 55% resulted in longer retention times. We selected imipramine, another tricyclic tertiary amine, as an internal standard because of its structural similarity to DOX. Since the assay was intended for bioavailability studies in normal volunteers we were not concerned about the exogenous administration of imipramine interfering with DOX and NDOX determination.

The sensitivity achieved in this assay was 2 ng/ml which is adequate for assessing single-dose bioavailability or pharmacokinetics. Lower sensitivity could be conceivably achieved by injecting a larger aliquot of the reconstituted residue obtained after solid-phase extraction.

Because of the intended use of the assay in normal volunteers in bioavailability studies, who typically are required not to take any other drugs, we did not perform any extensive interference studies using other drugs. In evaluating potential

internal standards we determined that protriptyline, amitriptyline and nortriptyline do not interfere with the assay. Barring interference from co-administered drugs this assay should be useful for therapeutic drug monitoring of DOX and NDOX serum concentations in patients at steady state since serum concentrations rarely are less than 10 ng/ml and turn-around-time is short.

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