Journal of Chromatography, 375 (1986) 202—206
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

CHROMBIO 2898

Note

Quantitative determination of doxepin and nordoxepin in urine by high-performance liquid chromatography

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(First received April 24th, 1985, revised manuscript received October 10th, 1985)

Doxepin (I, Fig. 1), a triclyclic antidepressant, is useful in the treatment of endogenous depression and anxiety with associated depression [1] Doxepin undergoes N-demethylation in vivo to form an active metabolite, nordoxepin (II, Fig 1) [2]. Simultaneous determination of doxepin and nordoxepin in biological fluids is important in a comparative bioavailability study, as well as in the evaluation of clinical response

For the determination of I and II in plasma, several methods have been reported, including gas chromatography (GC) [3—7], GC—mass spectroscopy

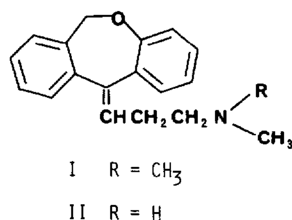

Fig 1 Structural formulae of doxepin (I) and its active metabolite nordoxepin (II)

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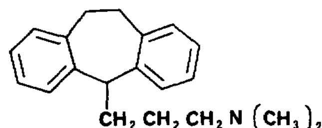
[8] and high-performance liquid chromatography (HPLC) [9—16]. Despite their high sensitivity, GC methods still suffer from insufficient resolution of the metabolite and parent drug without derivatization and require elaborate sample preparation involving derivatization. For these reasons, HPLC methods are now frequently used for the assay of I and II. These methods, however, lack adequate sensitivity for the plasma assay. Therefore, monitoring urinary excretion may be beneficial for comparative bioavailability study of different formulations.

This paper describes a selective HPLC method for assay of I and II in urine using imipramine (III, Fig. 2) as internal standard.



III

Fig 2 Structural formula of imipramine (III), the internal standard

EXPERIMENTAL

Materials

Doxepin hydrochloride (Pennwalt, Rochester, NY, U.S A), nordoxepin hydrochloride (prepared in-house from doxepin) and imipramine hydrochloride (Ciba, Summit, NJ, U.S.A.) were obtained from the Pennwalt Pharmaceutical Development Department. All chemicals used were analytical grade and the chromatographic solvents used were HPLC grade. Membrane filters (0.45 μm, Rainin Instrument, Woburn, MA, U S.A.) were used for filtration of the HPLC mobile phase. Disposable polypropylene centrifuge tubes (Evergreen Scientific, Los Angeles, CA, U.S.A) were utilized for extraction of samples.

Instrumentation

A modular high-performance liquid chromatograph was assembled consisting of a pump (Model 45, Waters Assoc , Milford, MA, U S A.), an autosample injector (WISP® Model 710B, Waters Assoc), a variable-wavelength UV spectrophotometer (DuPont, Wilmington, DE, U S A.), a recorder (Omni-Scribe® B-500 strip chart recorder, Houston Instruments, Austin, TX, U S A) and a power controller (Model 211, Autochrom, Milford, MA, U S.A.) Stainless-steel columns (12.5 × 0.32 cm I D) packed with hexyl reversed phase (Spherisorb® hexyl, 5 μm particle size, Deeside, U K , Hauppauge, NY, U.S.A.) at 550 bar were used for all analyses. A laboratory automation system (Model 3353 E, Hewlett-Packard, Avondale, PA, U.S.A) was used for quantitation and identification of chromatographic peaks A rugged rotator (Model PD-250, Glas-Col Apparatus, Terre Haute, IN, U.S.A.) was used for rotary mixing.

Chromatographic conditions

The mobile phase was 38% acetonitrile in 0 02 *M* phosphate buffer (mono-

basic) adjusted to pH 3.5 with 8 5% phosphoric acid The filtered and degassed mobile phase was pumped at a flow-rate of 1 ml/min through the column at room temperature The effluents were detected at 205 nm with 0.02 a.u f.s. sensitivity. The injection volume was 50 µl.

Preparation of urine standards

For each drug, 1 mg/ml (calculated as free base) stock solution was prepared in deionized water. From these stock solutions, working standard solutions containing I and II were prepared by dilution with water to concentrations ranging from 5 to 500 ng per 50 µl. Duplicate urine standards at the following concentrations were prepared by spiking drug-free human urine (1 ml) with an adequate volume (50—100 µl) of the working standard solutions 0, 10, 20, 50, 100, 250, 500, 1000 and 2500 ng/ml of urine. The internal standard solution of imipramine was prepared in water at a concentration of 2.5 µg/ml.

Preparation of validation samples

Validation samples at various concentrations of each drug were prepared by diluting a small volume (50—100 µl) of the standard solutions of doxepin and nordoxepin with drug-free human urine Two sets of triplicate samples at each concentration were prepared by pipetting 1 ml of the urine sample into coded tubes. One set of the samples was analyzed upon preparation, and the other set was kept frozen in a laboratory freezer for three weeks prior to assay

Extraction procedure

To 1 ml of urine were added 0.1 ml of the internal standard and 0.2 ml of 1 *M* sodium hydroxide to adjust the mixture to pH > 12. After vortexing, the sample was extracted with 7 ml of 2% *n*-butanol in hexane by rotomixing for 20 min. Following centrifugation at approximately 900 *g* for 5 min, the aqueous layer was frozen in a dry ice—acetone bath. The top organic layer was decanted and extracted with 0.2 ml of 0.1 *M* hydrochloric acid by rotomixing for 15 min After centrifugation at 900 *g* for 5 min, the organic layer was removed by aspirating An aliquot (50 µl) of the acid extract was analyzed by HPLC at room temperature as described under *Chromatographic conditions*

Quantitation

The peak-height ratios of each drug to the internal standard were obtained from the urine standards with the aid of a laboratory automation system The ratios were analyzed by linear regression with respect to their concentrations in the urine standards. The concentrations of I and II in the validation samples were determined by inverse prediction from the linear regression of the standards. The minimum quantifiable level was determined by linear regression [17].

RESULTS AND DISCUSSION

Typical chromatograms of drug-free and standard spiked urine extracts are presented in Fig. 3.

In contrast to commonly used mobile phases containing ion-pair reagents or organic amines as competing base, the mobile phase consisted of acetonitrile—

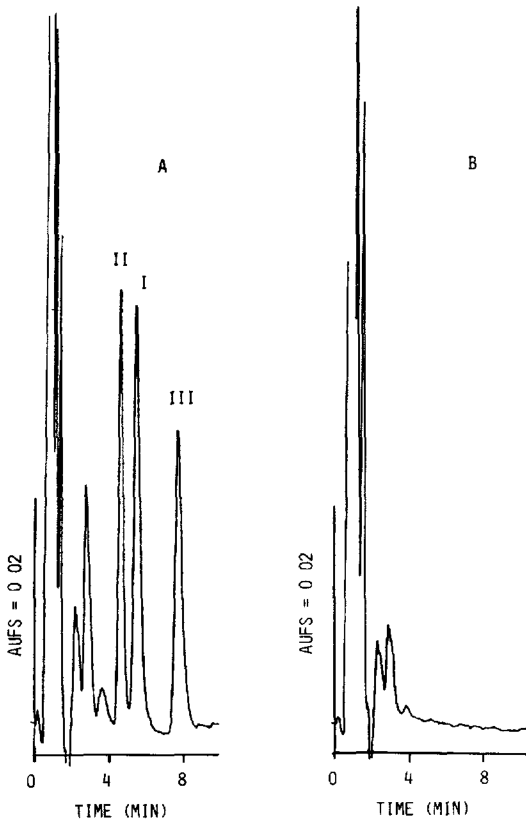


Fig 3 Chromatograms of urine extracts (A) Spiked urine with nordoxepin (150 ng/ml), doxepin (200 ng/ml) and imipramine, the internal standard, (B) control urine Peaks I = doxepin, II = nordoxepin, III = imipramine

TABLE I

SUMMARY OF DATA FROM ASSAY OF I AND II IN URINE

Nominal concentration (ng/ml)	Found concentration (mean ± S D , n = 3) (ng/ml)	R S D * (%)	Mean percentage difference**
Fresh samples			
40	43 3 ± 1 1	2 5	+8 3
90	82 7 ± 7 5	9 1	—8 1
200	190 7 ± 4 0	2 1	—4 7
800	764 0 ± 12 1	1 6	—4 5
1500	1313 0 ± 88 7	6 7	+12 5
Frozen samples			
40	46 0 ± 1 0	2 2	+15 0
90	81 7 ± 0 6	0 7	—9 3
200	172 0 ± 11 1	6 5	—14 0
800	779 7 ± 17 6	2 2	—2 5
1500	1361 7 ± 63 5	4 7	—9 2

*R S D (relative standard deviation, %) = (S D /mean) × 100
**Mean percentage difference = [(mean — nominal)/nominal] × 100

20 mM phosphate buffer at pH 3.5 A hexyl column was selected because the two drugs were separated well with adequate retention times. With the described chromatographic conditions, the typical retention times were 4.5, 5 5 and 7.4 min for I, II and III, respectively No interfering substances in blank urine were detected in the region of the drug peaks.

The sample preparation involved simple liquid—liquid extraction of drug and metabolite from basified (pH > 12) urine with 2% n-butanol—hexane followed by back-extraction into 0 1 M hydrochloric acid. Overall recoveries of both drugs were 84—100% in the concentration range 20—1000 ng/ml and about 90% for the internal standard

The regression analyses of the peak-height ratios of I and II to the internal standard versus their respective concentrations in the urine standards showed good linear relationships (r^2 > 0 998) The minimum quantifiable levels for both I and II with the lower five standards were 11—23 ng/ml of urine

The precision, which demonstrates reproducibility, and the accuracy of the method were evaluated by the assays of replicate fresh and frozen validation samples at the concentrations corresponding approximately to the lower and upper limit of the therapeutic ranges The results are summarized in Table I The similar assay results of fresh and frozen samples indicated no loss of drugs due to freezing the samples

Over the concentration range 40—1500 ng/ml, the precision, based on relative S D , ranged from 0 7 to 9 1%, and the accuracy, expressed as mean percentage difference from nominal, ranged from −14 to 15%. At the lower concentrations, the assay was less precise and accurate. However, the variation was still quite acceptable, considering the poor UV absorption of doxepin and nordoxepin.

In summary, a selective HPLC method was developed to quantitate doxepin and nordoxepin in urine. The method demonstrated suitable sensitivity for the detection of I and II in urine with no interference of biogenic substances

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