Journal of Chromatography, 344 (1985) 372—377
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2746

Note

Sensitive nitrogen—phosphorus capillary gas chromatographic assay for doxapram in premature infants

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(Received May 3rd, 1985)

Doxapram (I, Fig. 1) is currently being evaluated as a respiratory stimulant for the treatment of recurrent neonatal apnoea in premature infants [1]. In order to evaluate the pharmacokinetics of doxapram in these infants, successive blood samples are required. As only small blood volumes (less than 500 μl) can be obtained from these patients, the analytical procedure used to determine blood levels of drug must be very sensitive.

Several methods for doxapram analysis, including high-performance liquid chromatography (HPLC) [2], gas chromatography (GC) [3—5], and UV absorption [6], have been described. Of these available techniques, only GC,

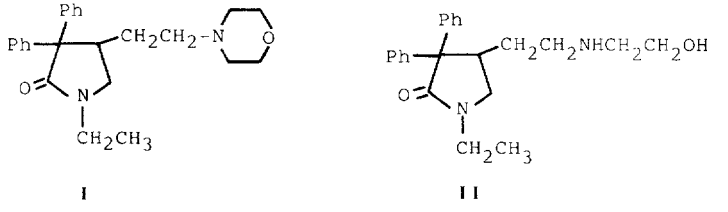


Fig. 1. Structures of doxapram (I) and its metabolite (II).

0378-4347/85/$03.30

linked to either a nitrogen—phosphorus detector or mass spectrometer, possesses the sensitivity and specificity desired for the analysis. Previous GC techniques, however, demonstrated that doxapram and its metabolites had poor GC characteristics, resulting in broad, poorly resolved chromatographic peaks [5]. Peak broadening is probably the result of adsorption of doxapram to the glass columns used. We have observed that doxapram at low concentrations (less than 10 μg/ml) is haphazardly adsorbed by glassware. This adsorption reduces both the sensitivity and reproducibility of the assay. Other disadvantages of previously published GC methods are the need for relatively large plasma volumes [3] and the necessity for a clean-up procedure when doxapram is analyzed in urine [4]. These disadvantages required us to seek a more appropriate analytical method which would provide adequate sensitivity and resolution and could be used routinely to assay doxapram in the plasma and urine of premature infants. Now described is a GC procedure, using a DB-5 fused-silica capillary column and a nitrogen—phosphorus detector, which offers all these qualities.

MATERIALS AND METHODS

Subjects, dosing and sample collection

Two premature newborn infants weighing 1.3 kg (patient A) and 1.4 kg (patient B) with creatinine clearance of 79 ml/min and 60 ml/min, respectively, required medication for their respiratory apnoea. They did not respond to theophylline and were therefore considered for doxapram therapy. The procedures and implications were explained to the parents and signed consent was obtained. The infants received constant intravenous doses of 2.0 mg/kg/h of doxapram · HCl (Dopram®, Robins Canada, Montreal, Canada). Once steady state was achieved in patient A, blood samples (500 μl) were withdrawn and collected, 6 h apart, into plastic polyethylene tubes containing EDTA as anticoagulant. The plasma was isolated from these samples by centrifugation. Urine, excreted over this 6-h period was also collected and stored in plastic containers. From patient B, consecutive blood samples (200 μl) were withdrawn every 2 h after intravenous infusion was discontinued. The plasma was again isolated from these blood samples as described above. Urine samples were not obtained from patient B. All plasma and urine samples were frozen at −26° C until analyzed.

Standards, reagents and sample preparation

Aqueous stock solutions of doxapram · HCl (Robins), 5.4 mg/ml, and the internal standard diazepam · HCl (Hoffmann-La Roche, Etobicoke, Canada), 0.11 mg/ml, were prepared in 1.5-ml microfuge tubes (Fisher Scientific, Fair Lawn, NJ, U.S.A.). The stock doxapram solution was suitably diluted to give concentrations ranging from 10 ng to 5 μg in 100 μl of plasma. The urine (2—5 μl) and plasma samples (20—100 μl), as well as the standards, were made up to a final volume of 200 μl with distilled water. To each standard and sample were added 25 μl of internal standard solution and 7 μl of concentrated perchloric acid. The precipitated protein was removed by centrifugation at 8730 *g* (Microfuge B, Beckman Instruments, Berkeley, CA, U.S.A.) for

4 min. Each supernatant was transferred to a clean microfuge tube and the perchloric acid neutralized with solid potassium bicarbonate (Fisher). The potassium perchlorate which formed was removed by centrifuging the samples for 4 min. The supernatant was again transferred to a 1.5-ml microfuge tube and potassium carbonate (Fisher) was added such that a small amount remained undissolved at the bottom of the tube. Following the addition of 1 ml of glass-distilled ethyl acetate (BDH, Toronto, Canada), each tube was vortexed for 5 min in a multitube vortex apparatus (IKA-Vibrax VXR, Terochem Labs., Edmonton, Canada) and then centrifuged for 4 min. The organic layer was retained and evaporated to dryness in a Savant Speed-Vac concentrator/evaporator (Emerston Instruments, Scarborough, Canada) and to the residue were added 50 μl of glass-distilled toluene (BDH). Of this solution 2 μl were injected onto the GC column.

Gas chromatography

The gas chromatograph was a Hewlett-Packard Model 5730A with a 18740B capillary column injector control and Model 3390A integrator-recorder (Hewlett-Packard, Palo Alto, CA, U.S.A.). The fused-silica capillary column (9 m × 0.32 mm I.D.) was coated (film thickness 1.0 μm) with DB-5 stationary phase (J & W Scientific, Rancho Cordova, CA, U.S.A.). The operating conditions were: injector, 250°C; detector, 250°C; column, 150—250°C (16°C/min). The gas flow-rates were: helium (carrier), 1 ml/min; hydrogen, 3 ml/min; air, 50 ml/min. The column head pressure was maintained at 0.85 bar.

Gas chromatography—mass spectrometry

Mass spectral analysis was carried out on a VG 7070E linked to a Varian Vista 6000 GC and PDP 11 data system (Analytech Instrumentation and Service, St. Laurent, Canada). The fused-silica column (30 m × 0.25 mm I.D.) was coated (film thickness 0.25 μm) with DB-1 stationary phase (J & W Scientific). The GC conditions were identical to those above except that the column temperature was 150—290°C (30°C/min).

RESULTS AND DISCUSSION

The chromatographic assay which has been described is sufficiently sensitive to allow the measurement of doxapram in successive plasma samples (20—100 μl) obtained from the same patient.

Various compounds were evaluated for their suitability as an internal standard. Diazepam was selected. The GC peaks of doxapram and the internal standard were well resolved and no contaminating peaks interfered with the assay (Fig. 2). The doxapram metabolite (II, Fig. 1) was also resolved from the doxapram peak (Fig. 2). The authenticity of the metabolite was confirmed by recording and interpreting its mass spectrum. Diagnostic ions were present at *m/z* 352 (molecular ion, 11% relative abundance), 334 (11%), 321 (100%), 291 (19%) and 278 (37%). Appropriate structures for these ions are suggested in Fig. 3.

The standard curve was found to be linear over the desired range of 10 ng to 5 μg per sample. Curves constructed on different days were always found to be

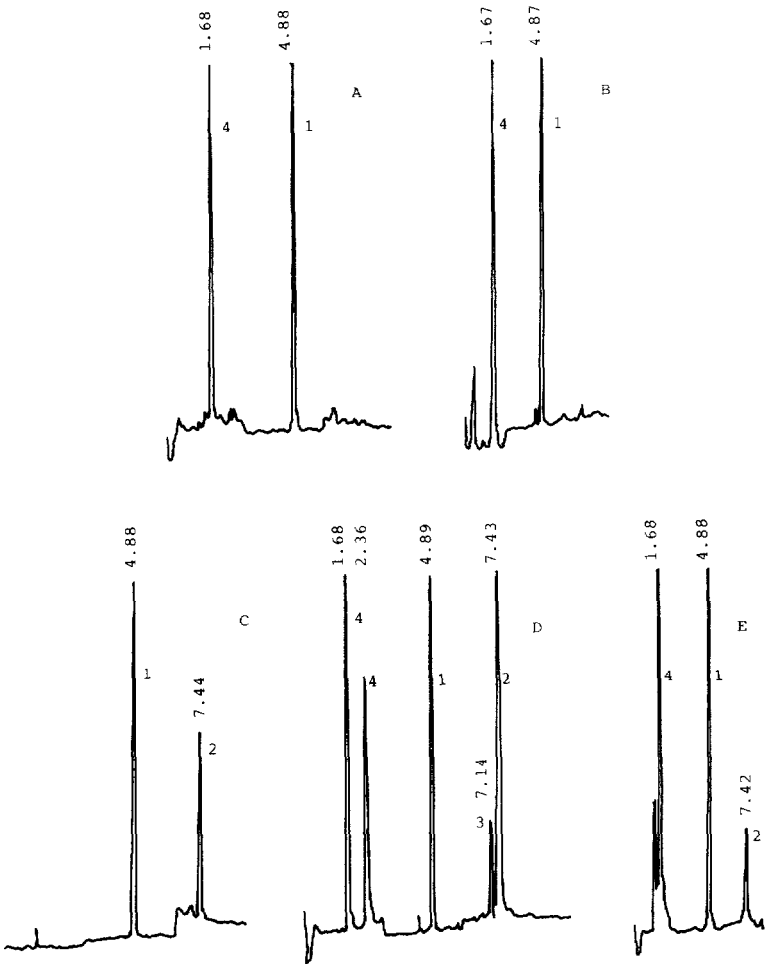
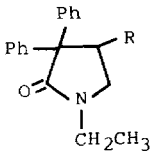


Fig. 2. Chromatograms of (A) blank plasma; (B) blank urine; (C) patient plasma sample (50 μl); (D) patient urine sample (30 μl); (E) plasma sample (100 μl) spiked with 2.5 ng doxapram. Recorder chart speed for traces A, C and D was 0.5 cm/min; for B and E 0.3 cm/min. The retention times are shown in min. Peaks: 1 = diazepam, internal standard; 2 = doxapram; 3 = doxapram metabolite; 4 = endogenous components.



m/z	R
352	$-CH_2CH_2\overset{+\cdot}{N}HCH_2CH_2OH$
334	$-CH_2CH_2\overset{+\cdot}{N}HCH{=}CH_2$
321	$-CH_2CH_2\overset{+}{N}H{=}CH_2$
291	$-CH{=}\overset{+}{C}H_2$
278	$-\overset{+}{C}H_2$

Fig. 3. Diagnostic mass spectral fragments of doxapram metabolite.

linear, although their slopes varied slightly from 0.405 to 0.413, due to changes in the response of the nitrogen—phosphorus detector. A typical best-fit line through the experimental points was described by $y = 0.405x - 0.007$ with a coefficient of correlation of 0.999. The minimum quantifiable concentration of doxapram in plasma samples was 1 ng in 100 μl, the maximum volume of plasma generally available. The coefficient of variance for triplicate samples (2 ng in 100 μl) was 5.3%.

The developed analytical procedure has been applied to the plasma and urine obtained from two premature infants infused with doxapram · HCl. Patient A was found to have doxapram concentrations of 4.1 and 5.3 μg/ml in plasma samples taken 6 h apart. The total quantity of doxapram excreted in urine over this period was 4.72 mg indicating a urinary excretion of 0.79 mg/h. The calculated total body clearance and renal clearance were found to be 8.86 and 2.79 ml/min, respectively. The plasma doxapram concentration—time curve obtained from patient B after withdrawal of the infusion is shown in Fig. 4. Doxapram is rapidly eliminated from plasma once infusion is discontinued. Levels of only 11 ng in 50 μl of plasma were reached within 6 h. From this data, the half-life of the terminal phase (β) was calculated to be approximately 2 h.

The reported method allows for the routine analysis of doxapram in as little as 2—5 μl of urine and 20—100 μl of plasma. This procedure is particularly attractive considering the difficulty encountered in obtaining sufficient biological samples from low-birth-weight infants. Doxapram was well resolved

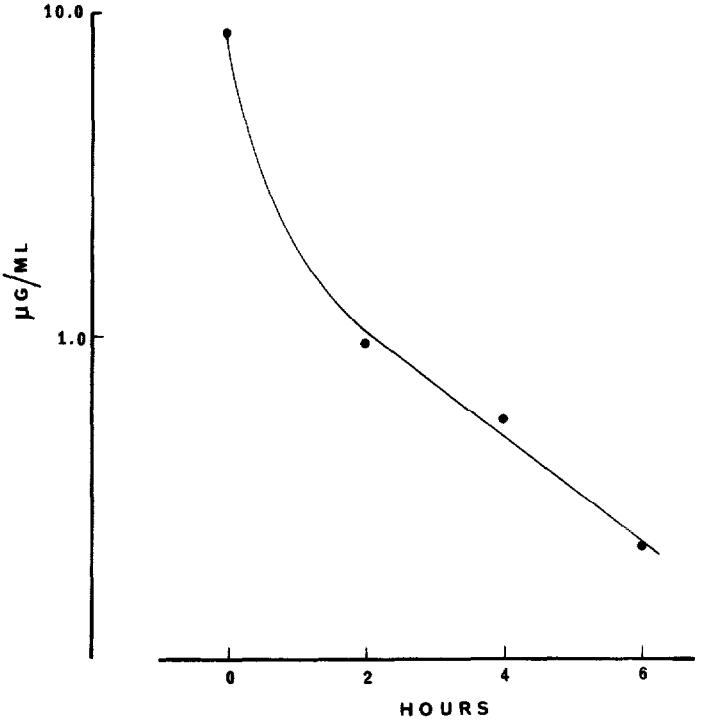


Fig. 4. Plasma concentration—time curve for doxapram in patient B after discontinuation of 2 mg/kg/h intravenous infusion.

from its metabolite and other endogenous plasma components. Since no additional clean-up procedure was required prior to extraction of the plasma or urine, the actual preparation time is quite short. The selection of an appropriate column seems to be vital for the GC assay procedure. We have also tried a 10-m DB-17 and 30-m DB-1 (J & W Scientific) and found both required excessively high temperatures for the elution of doxapram.

This assay procedure is suitable for therapeutic drug monitoring or for the study of the pharmacokinetics of doxapram in these infants. By establishing the therapeutic drug levels as well as excretion rate of doxapram, a safer and more effective therapy may be achieved. We are currently using this procedure to evaluate the kinetics of this drug in premature infants.

ACKNOWLEDGEMENT

An Alberta Heritage Foundation for Medical Research Studentship to G.A.T.-B. is gratefully acknowledged.

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