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QUANTITATION OF DOXAPRAM IN BLOOD, PLASMA AND URINE

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SUMMARY

Methods for the quantitation of doxapram in blood, plasma and urine have been developed. Following extraction, gas—liquid chromatography was used to separate doxapram from basic metabolites. Doxapram was detected by mass spectrometry for blood and plasma assays, and by flame ionisation for urine assays. The limit of reliable quantitation in blood and plasma was 10 ng and in urine 500 ng, the coefficients of variation being 6.37%, 1.72% and 2.31% respectively. To illustrate the clinical applicability of the assay methods, plasma, blood and urine levels were monitored in a premature newborn following an intravenous infusion of doxapram.

INTRODUCTION

Doxapram [1-ethyl-4-(2-morpholino-ethyl)-3,3-diphenyl-2-pyrrolidinone] is a central nervous system stimulant, acting primarily on the respiratory centres of the brain stem [1, 2]. Doxapram is currently being used in a clinical trial at the Women's Hospital, Sydney, Australia, to evaluate its efficacy in the treatment of recurrent neonatal apnoea in the premature newborn. One aspect of this trial was the determination of the pharmacokinetics of doxapram.

Previous analytical methods for the analysis of doxapram included oxidation of doxapram and doxapram-like materials to the benzophenone and quantitation by UV spectrophotometry [3]. A high-performance ion-exchange chromatographic method using A-5 resin has been used but was unable to separate doxapram from three basic metabolites [4].

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A rapid method for the analysis of doxapram in plasma has been reported using gas chromatography (GC) and a nitrogen-sensitive detector [5]. Data were not presented for the concentration range 0.01—0.75 μg per 100-μl sample of blood or plasma, which represents the levels expected in premature newborn.

In order to quantitate doxapram at the low levels present in blood and plasma, an assay based on gas chromatography—chemical ionisation mass spectrometry (GC—MS) with selective ion monitoring was developed. For the quantitation of doxapram in urine, where high sensitivity was not necessary, a GC method using flame ionisation detection (GC—FID) is described.

EXPERIMENTAL

Standards and reagents

Doxapram hydrochloride (Dopram®, A.H. Robins, Sydney, Australia) in multiple-dose vials (20 mg/ml) was used throughout the clinical trial. Doxapram hydrochloride was used as a primary standard (A.H. Robins). Dextromoramide [(+)-1-(3-methyl-4-morpholino-2,2-diphenylbutyryl)pyrrolidine] as the bitartrate salt, in single-dose ampoules, 5 mg/ml (F.H. Faulding, Adelaide, Australia) was used to prepare the internal standard solutions. All reagents and solvents were analytical reagent grade. Anaesthetic diethyl ether B.P. was distilled prior to use.

Qualitative analysis of urine

A cross section of urine samples from patients were extracted and analysed by GC—MS.

Extraction. Urine (3 ml), sodium hydroxide (0.5 ml, 5 *M*) and diethyl ether (10 ml) were placed in a 15-ml glass stoppered centrifuge tube, mixed for 2 min using a vortex mixer and centrifuged for 10 min at 1500 *g*. The diethyl ether layer was transferred to a second 15-ml glass stoppered centrifuge tube and hydrochloric acid (2 ml, 0.1 *M*) added. The mixing and centrifugation steps were repeated, the diethyl ether layer was discarded and sodium hydroxide (0.5 ml, 5 *M*) and diethyl ether (10 ml) were added to the aqueous phase. The mixing and centrifugation steps were repeated and the diethyl ether layer transferred to an evaporation tube (a 15-ml glass tube with a 100-μl capillary tube at the base). The diethyl ether was evaporated to dryness by immersion of the evaporation tube in a water bath at 40°. The tube was then stoppered and placed in ice to condense the diethyl ether vapour remaining in the tube. This evaporation—condensation procedure was then repeated until approximately 10 μl of diethyl ether remained. The sample was then analysed by GC—MS.

GC—MS. GC—MS results were obtained on a Finnigan 3200 quadrupole mass spectrometer fitted with a chemical ionisation source and interfaced to a Finnigan 9500 gas chromatograph. Data acquisition and processing were carried out on-line using a Finnigan 6110 data system. A pan coating method [6] was used to coat Gas-Chrom Q (100—120 mesh) with OV-225 (both from Applied Science Labs., State College, Pa., U.S.A.) using chloroform as the solvent.

A glass column (75 cm × 2 mm I.D.) was packed with 1% OV-225 and conditioned at a temperature of 250° and a methane flow-rate of 20 ml/min over-

night. GC—MS operating conditions: injection port, column, separator oven and transfer line temperatures were 250°, 235°, 260° and 260° respectively, methane (used as a carrier gas and chemical ionisation gas) flow-rate 20 ml/min; source pressure 1 torr; mass range scanned 100—450; scan time 3 sec.

Quantitative analysis of urine

Doxapram in urine was quantitated using a method based on GC—FID.

Extraction. Urine (0.5—2.0 ml), internal standard (dextromoramide 10 μg/100 μl in 0.1 *M* hydrochloric acid), sodium hydroxide (0.5 ml, 5 *M*) and diethyl ether (10 ml) were placed in a 15-ml glass stoppered centrifuge tube and the extraction procedure in the previous section was followed. Approximately half the ethereal concentrate was injected into the chromatography system.

GC—FID. A Hewlett-Packard gas chromatograph Model 5710A equipped with a flame ionisation detector was used. Resolution was achieved on a 75 cm × 2 mm I.D. glass column packed with 1% OV-225 on Gas-Chrom Q (100—120 mesh). The column was conditoned at 250° and a nitrogen flow-rate of 60 ml/min overnight. GC—FID operating conditions were: injection port, column and detector temperatures 250°, 230° and 250° respectively; nitrogen, air and hydrogen flow-rates 60, 240 and 60 ml/min respectively.

Calibration and reproducibility. Known quantities of doxapram hydrochloride (0.5—10 μg) were added to blank urine (0.5—2 ml) and assayed as above. Calibration curves were constructed by plotting the peak height of doxapram to dextromoramide against the amount of added doxapram. Reproducibility was determined by six replicate analyses at the upper (10 μg) and lower (0.5 μg) limits of the calibration range.

Quantitative analysis of blood and plasma

Doxapram in blood and plasma was quantitated using a method based on GC—chemical ionisation mass fragmentography.

Extraction. Blood or plasma (50—100 μl), internal standard (dextromoramide 500 ng/500 μl in 0.1 *M* hydrochloric acid), water (1.4 ml), sodium hydroxide (0.5 ml, 5 *M*) and diethyl ether (10 ml) were placed in a 15-ml glass stoppered centrifuge tube, mixed for 2 min (vortex mixer) and centrifuged for 10 min at 1500 *g*. The diethyl ether layer was transferred to an evaporation tube and evaporated as described above to approximately 10—15 μl. Half this concentrate was analysed.

GC—MS. The Finnigan system described in the section on the qualitative analysis of urine was used except that it was operated in the mass fragmentographic mode and column temperature was increased to 250°. The protonated molecular ions of doxapram (*m/e* 379) and dextromoramide (*m/e* 393) were monitored during the GC—MS run. Peak areas were determined using the Finnigan 6110 data system.

Calibration and reproducibility. Known amounts of doxapram hydrochloride (10—1000 ng for plasma, 10—500 ng for blood) were added to blood or plasma (50—100 μl) and assayed as described above. Calibration curves were constructed by plotting the peak area ratio of doxapram to dextromoramide against the amount of added doxapram. Reproducibility was determined by six

replicate analyses at the upper and lower limits of the calibration curve. Calibration plots were constructed on each day when patient samples were analysed.

Clinical protocol

Doxapram hydrochloride was administered to a premature newborn estimated gestational age 27 weeks, postnatal age 0.5 days, who had developed a recurrent neonatal apnoea. The doxapram was administered as a constant rate intravenous infusion at a rate of 2.4 mg/kg/h for 25 h, followed by an increase in dose rate to 3.8 mg/kg/h for 13.5 h. Blood samples (approximately 0.2 ml) were obtained from heel pricks and collected in heparinized tubes prior to and during the infusion, and for 48 h after cessation of infusion. Blood (50—100 μl) was measured directly into a 15-ml glass tube and the balance of the sample centrifuged and the plasma (50—100 μl) measured into a 15-ml glass tube. Urine samples were collected prior to and during the infusion, and for 48 h after cessation of infusion. The blood, plasma and urine samples were stored at −20° until analysed.

RESULTS AND DISCUSSION

Several chromatographic systems and extraction procedures were investigated in preliminary work attempting to quantitate doxapram in biological fluids by GC—FID. It soon became apparent that a clean-up procedure was required to remove neutral endogenous compounds extracted by diethyl ether. The main problem, thereafter, was the selection of a suitable chromatography

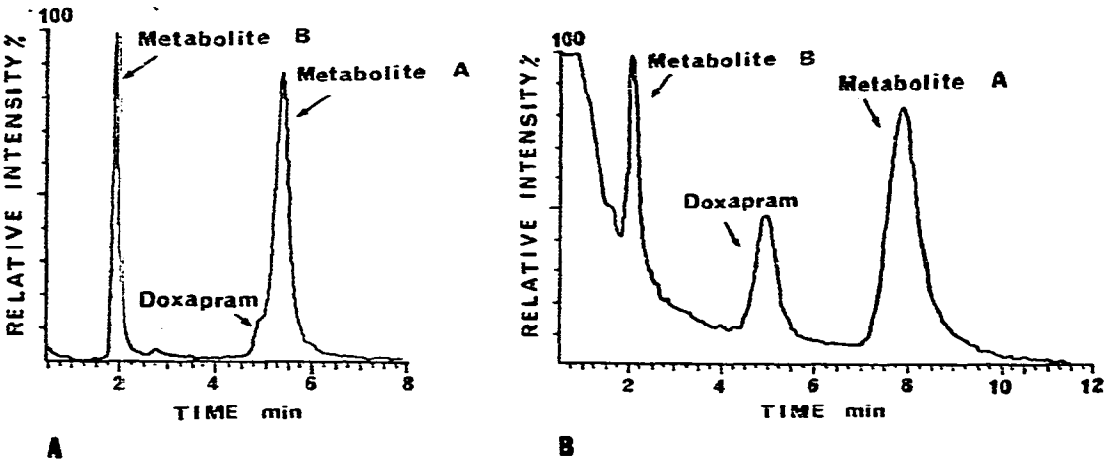


Fig. 1. Chemical ionisation, total ion current chromatograms of urine extracts from a premature newborn after the administration of doxapram. (A) Separation on 3% OV-17 on Gas-Chrom Q, 100—120 mesh; column, 150 cm × 2 mm I.D. Conditions: injection port, column, separator oven and transfer line temperatures 270°, 260°, 270° and 270° respectively; methane flow-rate 20 ml/min. (B) Separation on 1% OV-225 on Gas-Chrom Q, 100—120 mesh; column, 75 cm × 2 mm I.D. Conditions: injection port, column, separator oven and transfer line temperatures, 250°, 235°, 260° and 260° respectively; methane flow-rate 20 ml/min.

system to resolve doxapram from possible basic metabolites of doxapram which had been identified previously in urine in dogs [4]. Unfortunately, authentic samples of these compounds were not available to us and so to verify the authenticity and homogeneity of peaks quantitated as doxapram, GC—MS was used to analyse the eluent from several chromatography systems. A 3% OV-17 column was investigated initially since its use to quantitate doxapram in plasma had been reported [5]. As Fig. 1A illustrates, however, the OV-17 system was not suitable for the analysis of urine since it did not resolve doxapram from a metabolite whose chemical ionisation mass spectrum (Fig. 2B) indicates the structure assigned to metabolite A. The chemical ionisation mass spectrum of the only other urinary metabolite observed in this system is presented in Fig. 2C. There was no significant difference between the chemical ionisation mass spectrum of authentic doxapram and that of doxapram extracted from patient's urine and chromatographed on a 1% OV-225 system. Fig. 1B illustrates the chromatogram of a urine extract on this OV-225 system. Therefore, an OV-225 column was used for subsequent analyses of blood, plasma and urine.

It was decided to develop a method based on GC with chemical ionisation mass fragmentography because: (a) the volumes of blood and plasma available for analysis were small (50—100 μl), (b) the concentrations in some of these samples were expected to be low and (c) the base peak in the chemical ionisation mass spectrum of doxapram was the protonated molecular ion. Dextromoramide was used as internal standard because of its molecular similarity to doxapram and consequently for its mass spectral and chromatographic characteristics (Figs. 2A, 2D and 4B).

A representative mass fragmentogram of ions monitored in a patient's blood sample is illustrated in Fig. 3B. Similar mass fragmentograms were obtained in plasma and no interfering peaks from blank samples were observed (Fig. 3A). The calibration plots obtained from blood and plasma were linear and passed through the origin. The coefficients of variation for six replicate extractions of doxapram from blank blood and plasma are listed in Table I.

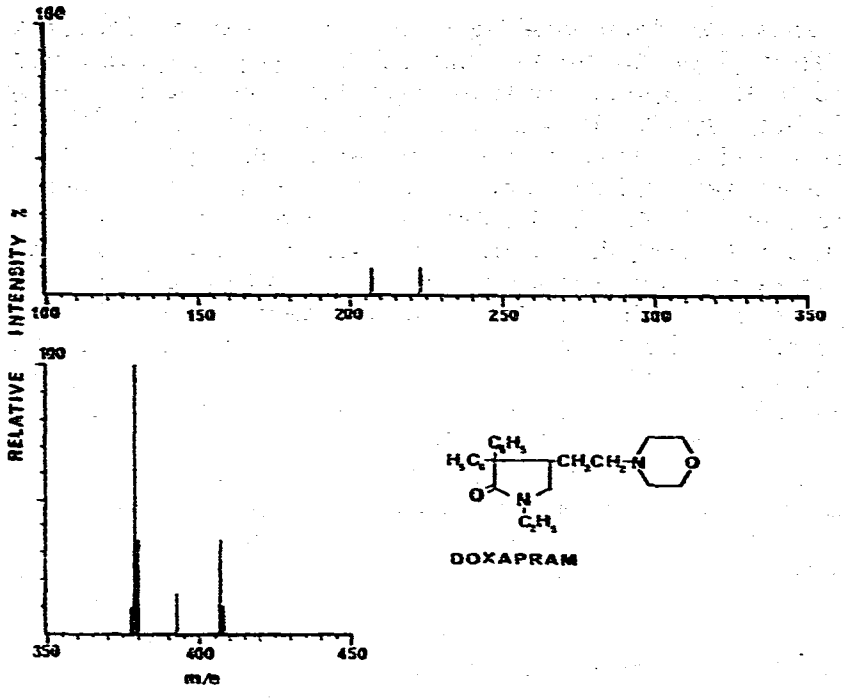
In the analysis of the urine samples where high sensitivity was not necessary, GC—FID was used. A typical chromatogram is illustrated in Fig. 4. A chromatogram resulting from the analysis of a blank urine sample is also shown. The calibration plots obtained were linear and passed through the origin. The coeffi-

TABLE I

REPRODUCIBILITY RESULTS RECORDED AT THE EXTREMITIES OF THE CALIBRATION RANGE FOR THE METHODS

Six replicate extractions were carried out.

Biologic fluid	Method of analysis	Doxapram HCl (μg)	Coefficient of variation (%)
Blood	GC—MS	0.010	6.37
		0.500	4.29
Plasma	GC—MS	0.010	1.72
		1.000	1.70
Urine	GC—FID	0.500	2.31
		10.000	2.42


RELATIVE INTENSITY %
100
150
200
250
300
350
m/e
400
450
DOXAPRAM
A

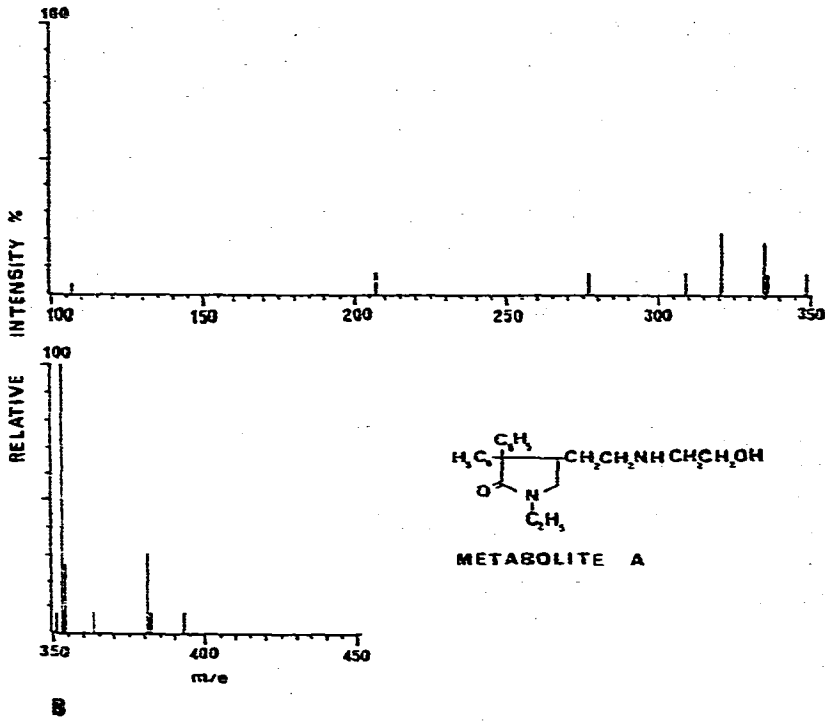

RELATIVE INTENSITY %
100
150
200
250
300
350
m/e
400
450
METABOLITE A
B

Fig. 2.

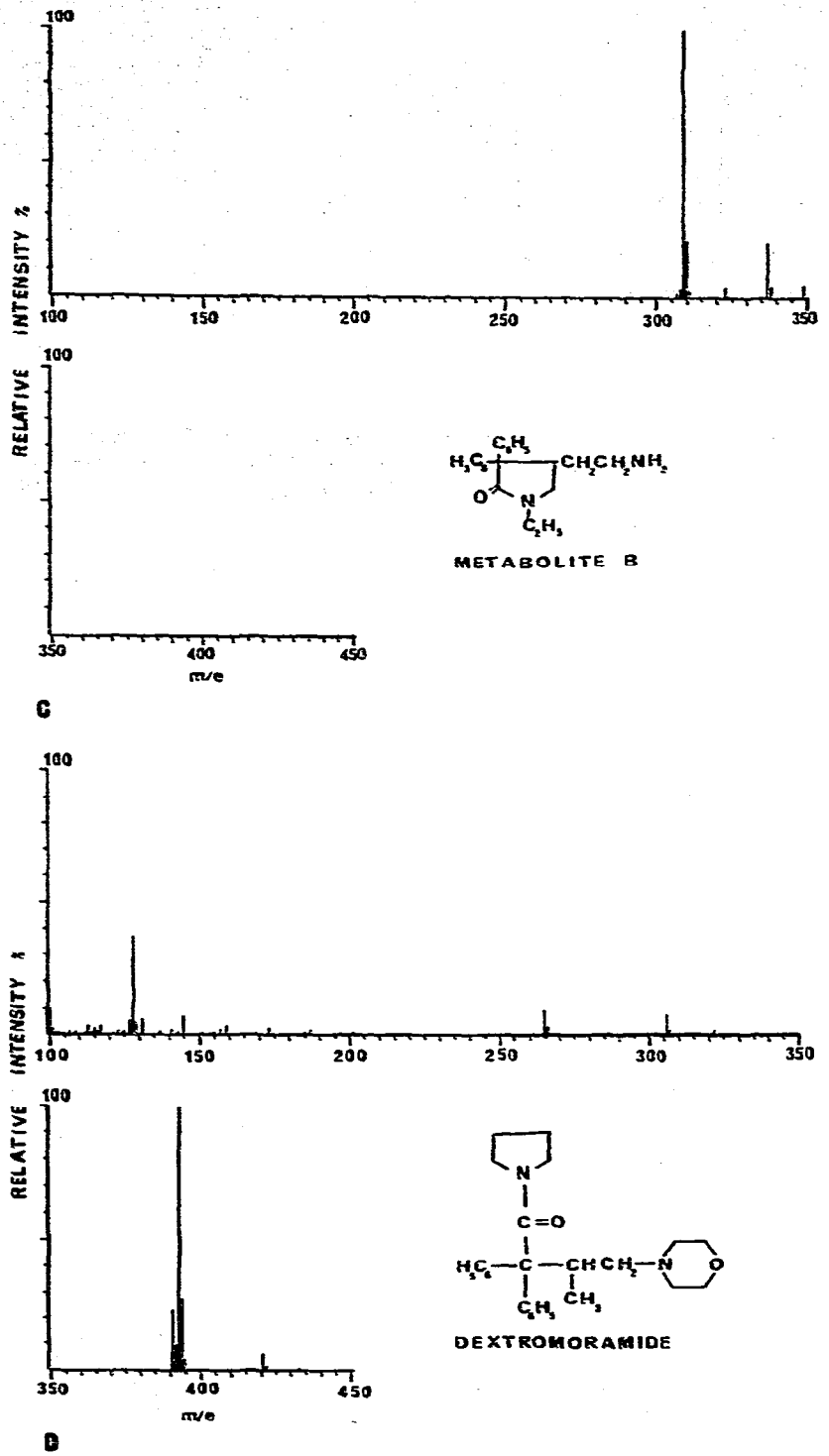


Fig. 2. Chemical ionisation mass spectra of (A) doxapram; (B) metabolite A, (C) metabolite B and (D) dextromoramide, obtained from a urine extract from a premature newborn receiving doxapram (the sample was spiked with internal standard dextromoramide). Separation was achieved on 1% OV-225 (GC–MS conditions, see text).

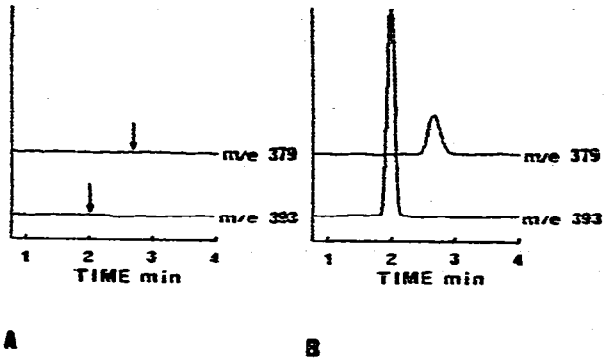


Fig. 3. Mass fragmentograms of blood samples from a premature newborn. The protonated molecular ions of doxapram (*m/e* 379) and dextromoramide (*m/e* 393) were monitored. (A) Blank blood sample; (B) blood sample containing 87 ng doxapram hydrochloride (GC—MS conditions, see text).

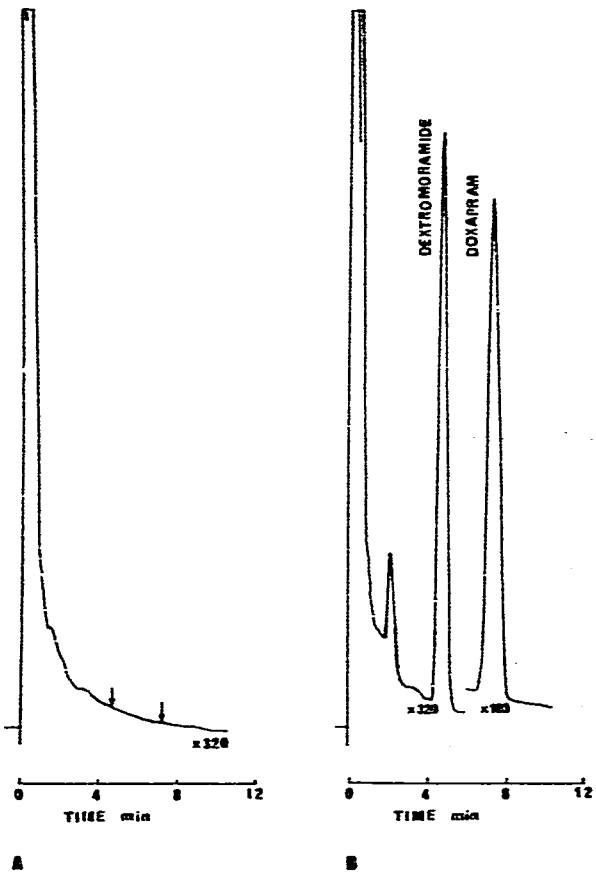


Fig. 4. Gas chromatogram of A, blank urine extract and B, urine extract from premature newborn receiving doxapram. Separation was achieved on 1% OV-225 (GC—FID conditions, see text).

cient of variation for six replicate analyses of doxapram from blank urine containing known amounts of doxapram is listed in Table I.

Fig. 5 presents the plasma and blood levels of doxapram during and after cessation of an infusion of doxapram in a premature newborn of estimated gestational age 27 weeks, postnatal age 0.5 days. The half-life as determined from the post infusion data was 7.7 h in blood and 8.6 h in plasma. Urinary excretion data are presented in Fig. 5, 5.4% of the dose being excreted unchanged.

These quantitative methods have been applied successfully in a study of the pharmacokinetics of doxapram in premature newborns and these results will be presented elsewhere.

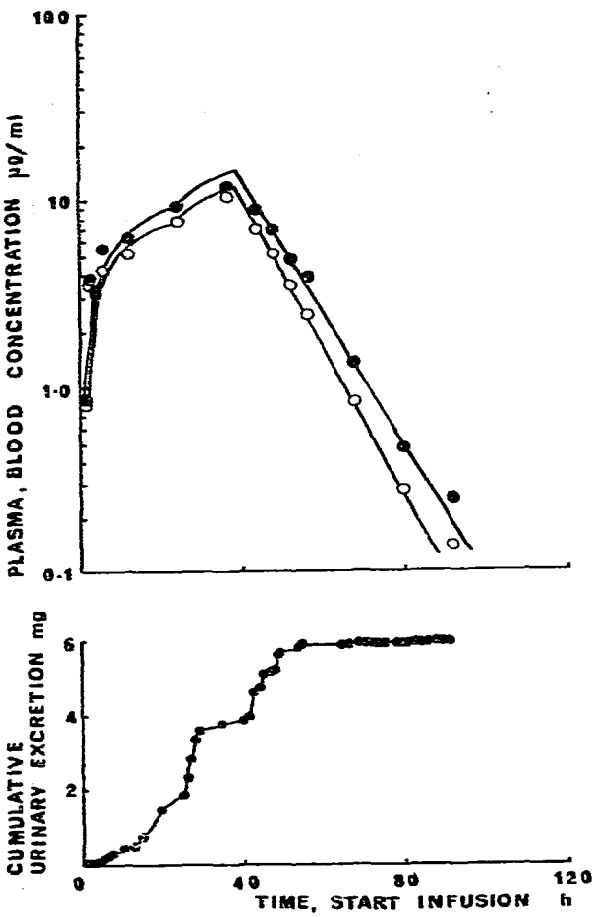


Fig. 5. (Top) blood (○) and plasma (●), and (bottom) urine data during and after cessation of an intravenous infusion of doxapram administered to a premature newborn.

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