Journal of Chromatography, 380 (1986) 455—461
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3169

Note

Determination of rubidazone and its metabolites in human plasma and urine by reversed-phase ion-pair high-performance liquid chromatography

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(First received December 31st, 1985; revised manuscript received March 15th, 1986)

Rubidazone (RBZ, Fig. 1) is an anthraquinone glycoside (anthracycline) used in the treatment of various acute leukemias (lymphoblastic and non-lymphoblastic) and solid tumours. It is generally administered when patients show resistance to the most commonly used anthracyclines: doxorubicin (DOX, Adriblastine®) and daunorubicin (DNR, Cerubidine®). Rubidazone is

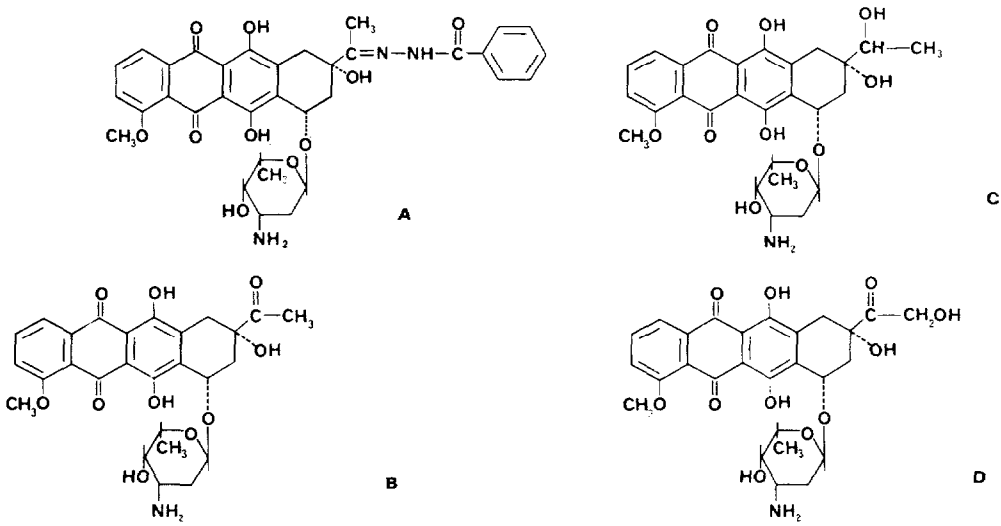

Fig. 1. Structural formulae of rubidazone (A), daunorubicin (B), daunorubinol (C) and doxorubicin (D).

0378-4347/86/$03.50

the benzoyl hydrazone derivative of daunorubicin, but it induces lower cardiomyopathy and bone-marrow toxicity than DNR or DOX [1].

Many studies on anthracycline pharmacokinetics in man (DOX, DNR, 4'-epi-DOX, aclacinomycin, etc.) have been reported but little information is available on RBZ, even though it has been used for many years. However, the metabolic pattern of RBZ is well known: the hydrolysis of the benzoyl hydrazone (RBZ) leads to DNR, which is further reduced to daunorubicinol (DOL). Baurain et al. [2, 4] have published a method for the assay of RBZ and its metabolites in mouse plasma and tissue, based on normal-phase high-performance liquid chromatography (HPLC) using a modified silica-packed column and fluorimetric detection, but the resolution of RBZ—DNR and DOX—DOL is too poor to allow sufficient accuracy at low levels. Furthermore, the chromatograms show peak asymmetry, especially for the later eluting peaks, and decreased sensitivity, making this method unsuitable for its application to pharmacokinetic studies in humans. Huhloven et al. [3] proposed a normal-phase HPLC method for the analysis of RBZ and its metabolites in human plasma, using a slightly different column, but the claimed separation of the four main components can rarely be obtained with enough accuracy when analysing patient samples.

A fairly simple isocratic HPLC method for RBZ, DNR and DOL analysis in human plasma and urine, using a reversed-phase column, ion-pair reagent, fluorimetric detection and involving a rapid liquid—solid extraction step, is reported in this paper.

EXPERIMENTAL

Chromatographic system

The HPLC system consisted of a Beckman 114M solvent delivery system (Beckman Instruments, Berkeley, CA, U.S.A.) fitted with a Rheodyne 7125 sample valve equipped with a 50-μl loop, and a Fluoromat FS 950 fluorimeter (Kratos, Schoeffel Inst. Div, Westwood, NJ, U.S.A.) using interference filters of 250 and 580 nm for excitation and emission wavelengths, respectively.

The column was 15 cm × 3.9 mm I.D., packed with 5-μm end-capped RP-18 sorbent (Nova-Pak C_{18}, Waters Assoc., Milford, MA, U.S.A.) and used at ambient room temperature.

The mobile phase consisted of acetonitrile—0.1 *M* glycine buffer (pH 7.6)—Pic B7 reagent (32:66:2) maintained at a flow-rate of 1.2 ml/min (ca. 100 bar pressure); the mobile phase was degassed by sonication using a "Bransonic" sonicator.

Reagents

All reagents were of analytical grade. Acetonitrile, methanol and chloroform were purchased from Merck (Darmstadt, F.R.G.); sodium chloride was obtained from Sigma and glycine from Merck; heptanesulphonic acid (Pic B7 reagent) and C_{18} cartridges (Sep-Pak) were from Waters Assoc.

All glassware was soaked in detergent for 1 h and rinsed thoroughly with bi-distilled water, twice.

Rubidazone, daunorubicinol, daunorubicin and doxorubicin hydrochlorides were kindly supplied by Rhône Poulenc Santé (Vitry, France).

Standard solutions and blood samples

Plasma standard solutions were obtained from a standard solution of RBZ, freshly prepared every day, at a concentration of 40 μg/ml in 0.1 *M* glycine buffer (pH 7.6). Standard stock solutions of DOL, DNR and DOX were prepared at a concentration of 200 μg/ml in methanol. Drug-free venous blood was obtained from healthy human subjects. Blood was collected in glass tubes containing sodium heparin, centrifuged at 2000 *g* for 15 min and the plasma was stored at −20° C until assayed.

Eight subjects suffering from solid tumours have been given RBZ intravenously as a single bolus dose of 4 mg/kg. Plasma concentrations were followed over 48 h and urine was collected over 72 h. The results obtained for one patient are reported in Tables I and II.

TABLE I

RBZ, DOL AND DNR CONCENTRATIONS IN PLASMA OF PATIENT AFTER A SINGLE INTRAVENOUS DOSE OF RBZ AT 4 mg/kg

The measured percentages of DNR include the in vitro conversion of RBZ into DNR and the possible formation of DNR from RBZ in vivo.

	Time of sampling after dose (h)					
	0	0.25	1	12	24	48
RBZ concentration (ng/ml)	0	15482	1303	138	113	59
DOL concentration (ng/ml)	0	71	28	32	28	18
DNR (percentage of RBZ fluorescence)	0	13.7	17	27	14.6	12.9

TABLE II

DNR AND DOL CONCENTRATIONS IN URINE OF THE SAME PATIENT

	Time of sampling after dose (h)			
	0	24	48	72
DOL concentration (ng/ml)	0	261	28	12
DNR concentration (ng/ml)	0	396	110	28

Extraction procedure

A liquid—solid extraction using adsorbent cartridges containing a plug of reversed-phase Sep-Pak C_{18}, has been investigated for the clean-up of plasma samples [5]. The Sep-Pak cartridge was activated by passing 3 ml of methanol, followed by 3 ml of methanol—water (1:1) through it. The pH conditions

were reached by rinsing the cartridge with 5 ml of 0.1 *M* glycine buffer (pH 7.6). The plasma sample (2 ml) was then carried through the Sep-Pak cartridge and washed with 1 ml of glycine buffer. A 4-ml volume of chloroform—methanol (4:1) was passed through the cartridge so that the analyte was stripped off and the eluate collected in a 12-ml glass-stoppered conical centrifuge tube. The void-volume of the cartridge consisted of the first 0.4 ml of eluate, which was drawn off and discarded. The organic layer was immediately evaporated to dryness at 40° C under a gentle stream of nitrogen, and the residue was readily reconstituted by whirlimixing in a suitable volume of 0.1 *M* glycine buffer (pH 7.6). An 50-µl aliquot was injected into the HPLC system. For urine analysis, 50-µl aliquots were directly injected into the chromatographic system without any extraction step.

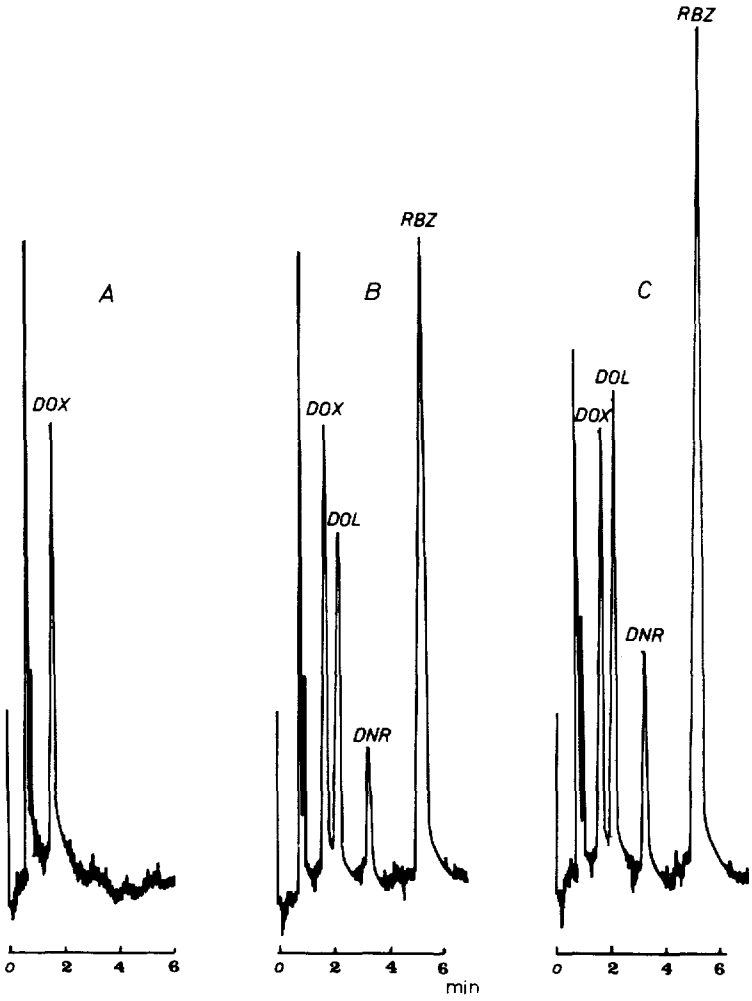

Fig. 2. Chromatograms of extracts from plasma spiked with 40 ng/ml DOX as internal standard. (A) Control drug-free plasma. (B) Plasma containing 100 ng/ml RBZ (12% hydrolysis into DNR) and 25 ng/ml DOL. (C) Patient's plasma 12 h after a single intravenous dose of RBZ at 4 mg/kg; RBZ = 138 ng/ml (18% hydrolysis into DNR); DOL = 32 ng/ml.

RESULTS AND DISCUSSION

The selectivity of the assay is demonstrated in Fig. 2, which displays chromatograms of a processed sample of drug-free plasma spiked with known amounts of RBZ and DOL and a human plasma sample from a clinical study. All of them were spiked with DOX as internal standard. No interference from endogenous human plasma components was observed at the retention times of

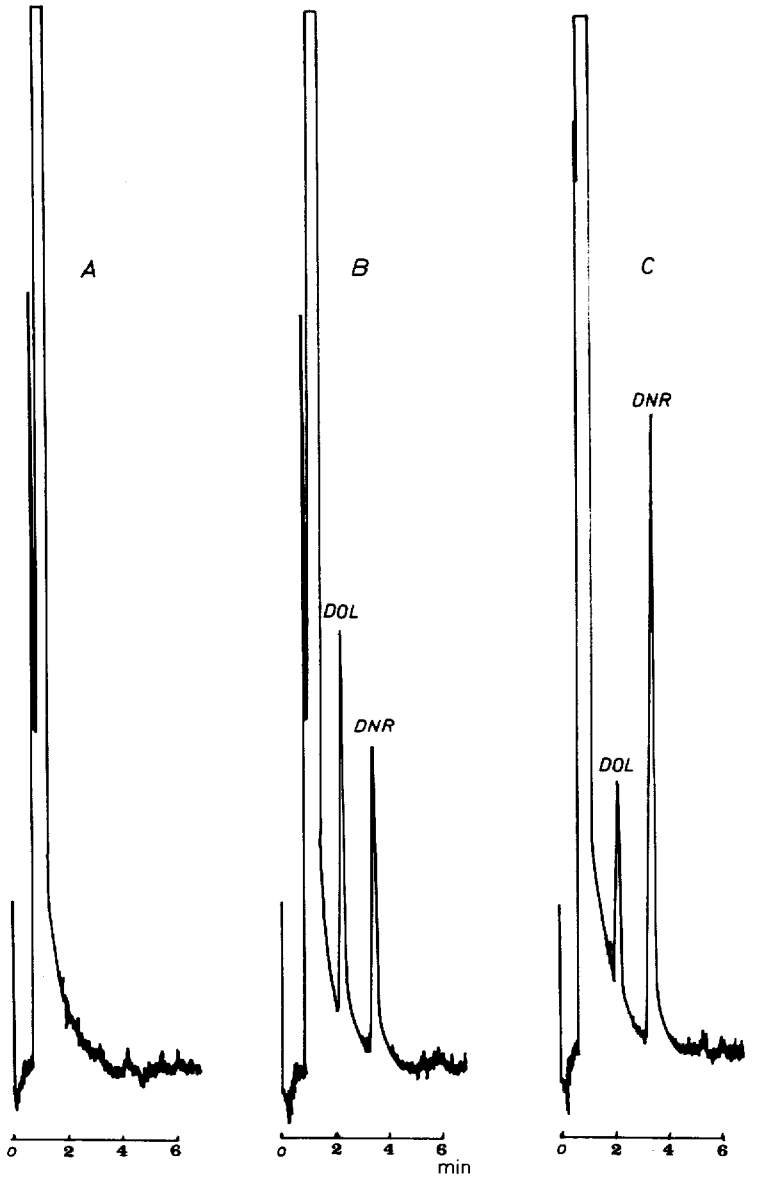

Fig. 3. Chromatograms of urine directly injected into the HPLC system. No internal standard was required. (A) Control drug-free urine. (B) Urine containing 50 ng/ml DNR and 50 ng/ml DOL. (C) Patient's urine 48 h after a single dose of RBZ at 4 mg/kg. No RBZ was found. DNR = 110 ng/ml and DOL = 28 ng/ml.

RBZ, DNR, DOL and DOX, which are 5.4, 3.4, 2.0 and 1.6 min, respectively. All these observations apply equally to the urine samples, which were directly injected into the HPLC system. The chromatograms are shown in Fig. 3.

Standard curve data were generated by analysing a series of standards. Data were analysed by linear regression (peak-height ratios versus plasma concentrations) using the reciprocal of the variance of the peak-height ratios as the weighting factor. The peak-height ratio of RBZ/internal standard is linearly related to its plasma concentration up to 15 μg/ml plasma, as indicated by the high correlation coefficient (r = 0.999) of a ten-point curve. Similar standard curves have also been generated for spiked urine samples. Excellent accuracy and precision were obtained.

Variations in the calibration curve from day to day were small, the coefficient of variation of the slope being 8.3%.

The reproducibility of the assay was estimated at two concentrations, 50 and 1000 ng/ml, with six determinations at each concentration. The mean values and the coefficients of variation were 48.5 ± 8.1% and 1021 ± 6.4%, respectively.

The limit of detection of the assay was set at a signal-to-noise ratio of 3:1 and was ca. 5 ng/ml when 2 ml of plasma were used, and 20 ng/ml for urine.

RBZ is rapidly hydrolysed into DNR at a pH different from 7.6. Its stability also depends on its concentration and on the ionic strength of the buffer used [2]. We have used a standard solution of RBZ at 40 μg/ml in 0.1 *M* glycine buffer (pH 7.6) at ambient room temperature. Under these conditions, 50% hydrolysis is reached after 33 h, which demands the standard solution of RBZ to be freshly prepared every day. The RBZ powder when dissolved in 0.1 *M* glycine buffer (pH 7.6) and injected directly into the chromatograph was found to contain 8—15% DNR. Since RBZ is metabolized into DNR in the organism, this has to be taken into account when determining endogenous and exogenous DNR.

CONCLUSION

The present method achieves complete separation of RBZ and its major glycoside metabolites DNR and DOL by using DOX as internal standard and without requiring gradient elution. A complete analysis can be performed in < 6 min without broadening of the later-eluting peaks or peak tailing. Sensitivity, accuracy and selectivity of the method allow pharmacokinetic

TABLE III

RECOVERYIES OF SEP-PAK EXTRACTION FOR DIFFERENT ANTHRACYCLINES

Concentration = 500 ng/ml for each anthracycline; volume of extracted plasma = 2 ml; n = 6.

	Recovery (%)	S.D.
RBZ	93.0	9.4
DOL	85.3	5.0
DNR	79.7	5.9
DOX	91.0	4.7

studies in humans by analysis of plasma and urine samples collected over 48 h after administration of the drug.

The extraction step consists of an attractive work-up procedure, which shows the following advantages: simplicity, efficiency and similar recoveries of various anthracyclines (Table III and ref. 6). Moreover, the speed of this procedure allows the make-up of twelve points of a pharmacokinetic study within one day, including the standard curve generation.

In summary, this technique is simple, easy to perform and relatively unexpensive for plasma and urine analysis.

ACKNOWLEDGEMENTS

We wish to thank Dr. F. Djebbar and Dr. F. Lavelle from Rhône Poulenc Santé, Courbevoie, France, for supporting this work.

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