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Note

Simultaneous determination of some radiosensitizing and chemotherapeutic drugs in plasma by thin-layer chromatography

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Hypoxic cell radiosensitizers, particularly misonidazole, are extensively used in radiotherapy. Since it has been demonstrated that misonidazole can enhance the cytotoxicity of some chemotherapeutic agents [1], recent attention has been directed to the possible therapeutic use of the radiosensitizing drugs in combination chemotherapy [2]. In order to assess the clinical potential of combining radiosensitizers with cytotoxic drugs, the simultaneous study of their pharmacokinetics could be of interest. Consequently, we have developed a thin-layer chromatographic (TLC) method for assaying at the same time some chemotherapeutic agents and radiosensitizers. The drugs that have been investigated are misonidazole (MIS), its metabolite desmethylmisonidazole (DEMIS), 1-methyl-2-nitro-5-vinylimidazole (L 8580), cyclophosphamide (CYP) and 5-fluorouracil (f-FU).

EXPERIMENTAL

Reagents

All chemicals and solvents were of analytical grade. CYP was obtained from Schering (Milan, Italy); MIS, DEMIS, ipronidazole (IPR), ornidazole (ORN), and 5-FU were from Hoffmann-La Roche (Basel, Switzerland). 2-Methyl-5-nitroimidazole (MNI) was supplied by Aldrich Europe (Beerse, Belgium), and L 8580 was from Lepetit (Milan, Italy). All drugs were used without further purification.

Stock standard solutions of the compounds to be assayed were prepared in methanol. All solutions were kept refrigerated in tin-foil-wrapped flasks and prepared fresh every two weeks. Working standard solutions were made by dilution to appropriate concentrations with methanol, and discarded after a single use.

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Extraction procedure

Sep-Pak C_{18} extraction. Plasma samples of 1 ml volume containing known amounts of CYP, MIS, and L 8580 or IPR [as internal standard (I.S.)] or alternatively known amounts of L 8580, CYP, and MNI (as I.S.) were treated with 1 ml of borate buffer (pH 10). The mixture was passed through a Sep-Pak C_{18} cartridge pre-wetted with 3 ml of methanol and 5 ml of buffer solution. The cartridge was washed with 1.5 ml of borate buffer, 1.5 ml of water, and 1.5 ml of methanol. This last fraction, which completely eluted the drugs from the cartridge, was vacuum evaporated to dryness keeping the temperature below 40°C. The residue was redissolved in 100 μl of methanol and 1-μl aliquots were chromatographed with the eluents indicated in Table III.

Solvent extraction. To 1 ml of plasma spiked with known amounts of 5-FU, L 8580 and ORN (as I.S.) or alternatively with MIS, DEMIS, 5-FU and L 8580 (as I.S.), CYP, L 8580 and MNI (as I.S.), and MIS, DEMIS, CYP and L 8580 (as I.S.), was added an equal volume of a saturated ammonium sulphate solution. The mixture was extracted twice with 8 ml of isopropanol—ethyl acetate (1:4, v/v). The extraction was performed by a mechanical shaker for 5 min. After centrifugation the organic phase was vacuum-evaporated to dryness and processed as described above.

Chromatographic procedure

A 1-μl sample was spotted on pre-coated layers of silica gel G-60 F_{254} (aluminium plates 0.25 mm thick, Merck, Darmstadt, G.F.R.) with a Camag (Muttenz, Switzerland) micro-applicator. The layers were pre-washed with the eluent mixture to be used. The TLC plates were developed in saturated (paper-lined) tanks at room temperature. After development, the layers were air-dried and the spots were quantitated by scanning densitometry by means of a Camag TLC/HPTLC 76500 scanner. The readings were performed at 320 nm for MIS, DEMIS, MNI, ORN, and IPR, and at 254 nm for 5-FU and L 8580. After this scanning, the plates were heated for 10 min at 260°C for detecting CYP as previously described [3], and then scanned at 254 nm. The standard curves were prepared by spotting 1 μl of working standard solutions and plotting peak areas against ng applied.

RESULTS AND DISCUSSION

Sep-Pak C_{18} extraction

On the basis of the good results obtained for the extraction of CYP from plasma buffered at pH 10 [3], the same procedure was utilized for extracting the other drugs. The results obtained showed a complete retention of CYP, MIS, L 8580, IPR and MNI by the cartridge. On the other hand, DEMIS and 5-FU were not retained. These two drugs were not retained even if the plasma was buffered at pH 4 or pH 6. As a consequence, we resorted to a solvent extraction from plasma for 5-FU and DEMIS. The recovery and reproducibility of the Sep-Pak C_{18} extraction method are presented in Table I.

Solvent extraction

5-FU was extracted from plasma as described by Min and Garland [4]

TABLE I

RECOVERY OF DRUGS FROM PLASMA AND REPRODUCIBILITY OF THE SEP-PAK C_{18} EXTRACTION

Drug	Amount added (μg/ml)	Recovery (%)	Reproducibility (6 samples)		
			Mean (μg/ml)	± S.D.	C.V. (%)
CYP	31.8	94.6	30.1	1.5	5.1
MIS	52.9	90.2	47.7	2.2	4.6
L 8580	36.1	98.7	35.6	1.1	3.2
IPR	55.7	83.1	46.3	2.2	4.8
MNI	20.2	97.7	19.7	0.8	4.0

TABLE II

RECOVERY OF DRUGS FROM PLASMA AND REPRODUCIBILITY OF THE SOLVENT EXTRACTION

Drug	Amount added (μg/ml)	Recovery (%)	Reproducibility (6 samples)		
			Mean (μg/ml)	± S.D.	C.V. (%)
5-FU	26.7	97.0	25.9	1.1	4.3
L-8580	32.5	101.3	32.1	1.6	4.9
ORN	41.1	101.6	40.5	1.5	3.8
MIS	54.4	94.2	51.3	2.3	4.4
DEMIS	32.9	86.4	31.7	1.6	5.0
CYP	37.9	93.8	35.6	1.1	3.2
MNI	27.9	99.8	27.8	1.1	3.9
IPR	43.0	45.4	—	—	—

utilizing, however, isopropanol—ethyl acetate (1:4, v/v) as the organic phase. In such a way the mean recovery obtained was very good, 97%. We did not perform the benzene wash because we found no effect on the baseline of the chromatogram of the blank plasma. The solvent extraction procedure was found to be suitable also for MIS, its metabolite DEMIS, L 8580, CYP, and ORN, but not for IPR. As a consequence, IPR cannot be used as internal standard when performing solvent extraction. The recovery and reproducibility of the solvent extraction method are presented in Table II.

Chromatographic procedure

The $R_F \times 100$ values given in Table III each represents the average of a minimum of three separate chromatographic runs. Under the conditions described under Experimental all R_F values were reproducible (C.V. = 4%). Fig. 1 shows typical chromatograms obtained from plasma samples spiked with different mixtures of the drugs under investigation. Also shown in the figure are the chromatograms of plasma blanks processed by both the extraction procedures. As can be seen, the drugs are well separated and free from interferences from biological components. The calibration graphs calculated

TABLE III

$R_F \times 100$ VALUES OF CHEMOTHERAPEUTIC AND RADIOSENSITIZING DRUGS IN VARIOUS SOLVENT SYSTEMS

Compound	Eluting solvent*			
	I	II	III	IV**
CYP	33	11	52	
MIS	41			36
DEMIS	23			13
L 8580	60	59	71	74
5-FU		19	18	26
MNI		25	33	
ORN		28		
IPR	60			

*Solvent systems used: I = ethyl acetate—dichloromethane—methanol (5:3:1, v/v); II = ethyl acetate—dichloromethane—methanol (15:10:1, v/v); III = dichloromethane—methanol (7.5:1, v/v); IV = chloroform—ethyl acetate—ethanol (7.5:7.5:1, v/v). Solvent run = 8 cm.
** Double development.

as a function of the amount applied to the layer were linear up to 2 μg of CYP, 1 μg of MIS, DEMIS, 5-FU and L 8580 at least (corresponding to 200 μg/ml for CYP, and 100 μg/ml for the other drugs). The detection limits were about 50 ng applied to the layer of CYP, and 10 ng of the other compounds; i.e. the sensitivity of the assay, using 1 ml of plasma, was 5 μg/ml for CYP, and 1 μg/ml for MIS, DEMIS, 5-FU and L 8580. These concentration ranges can obviously change depending on the volume of plasma assayed and/or the volume of solvent used to redissolve the residue after extraction.

CONCLUSIONS

The TLC method described for the simultaneous determination of CYP and 5-FU with some radiosensitizing drugs in plasma samples is simple, rapid and reliable. The usual levels of radiosensitizers in plasma to be analyzed are within the upper and the lower concentration limits reported above [5]. The proposed method is less sensitive for assaying CYP and 5-FU than the gas chromatographic methods commonly used, but equivalent to the electrochemical and high-performance liquid chromatographic (HPLC) techniques for assaying the radiosensitizers [5]. Nevertheless, the levels of CYP and 5-FU detectable by the TLC method cover the major part of the levels usually found in plasma [6—8]. The method allows the simultaneous determination of radiosensitizers and chemotherapeutic agents by two separate scans, yet showing an equivalent sensitivity for all the tested drugs. Analysis of the same preparation could be also performed by HPLC but in this case the sensitivity for CYP would be worse because of the low molar extinction coefficient of CYP even if the detection is performed at 200 nm [9]. Therefore, the method described here proves suitable for the study of the pharmacokinetics of radiosensitizers when given in combination with chemotherapeutic

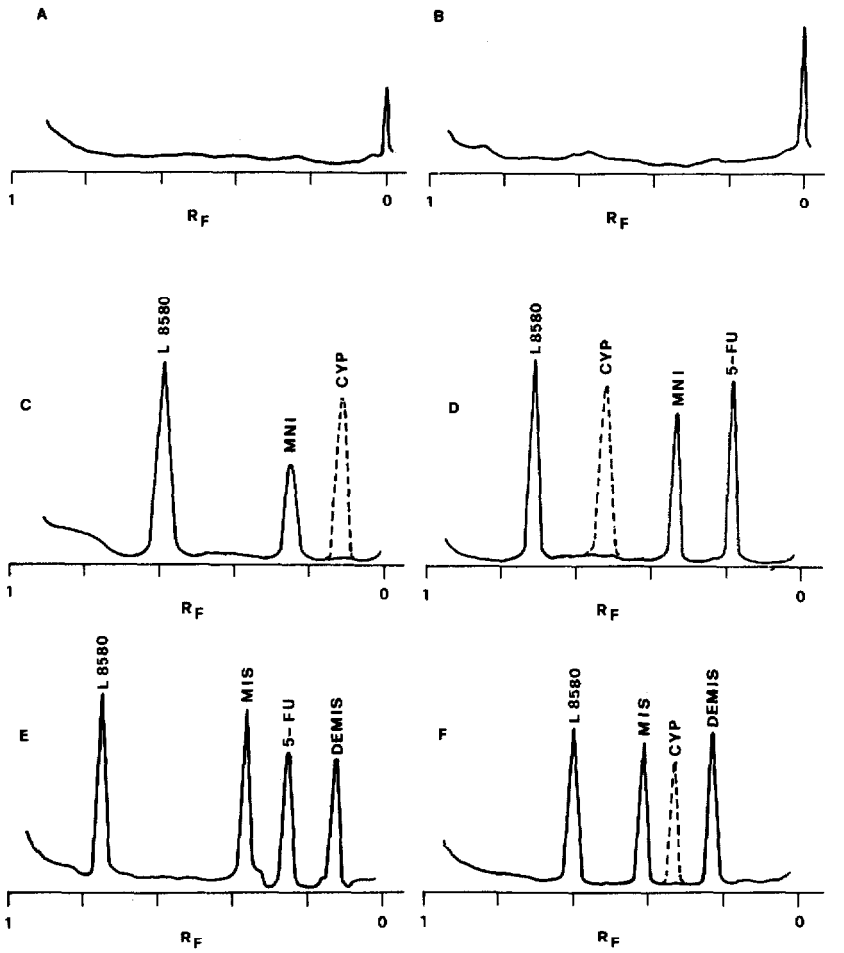

Fig. 1. Chromatograms of: (A) plasma blank from Sep-Pak C_{18} extraction; (B) plasma blank from solvent extraction; (C—F) plasma samples spiked with chemotherapeutic and radiosensitizing drugs. (- - - -), reading performed after heating at 260°C. Eluents used: I for A and F, II for B and C, III for D, IV for E. Abbreviations as in the text.

agents, or for studies which need short analysis times, and simple manipulations, especially when radiolabelled drugs are used or qualitative tests are required.

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