GLC Determination of Floxuridine in Plasma Using a Thermionic Nitrogen Detector

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Received March 18, 1977, from the *Laboratoria voor Medische Biochemie en voor Klinische Analyse, Faculteit van de Farmaceutische Wetenschappen, Rijksuniversiteit, Academisch Ziekenhuis, 135 De Pintelaan, 9000-Gent, Belgium.* Accepted for publication June 24, 1977.

Abstract □ A specific GLC method was developed for the determination of floxuridine in plasma using the thermionic nitrogen detector. The method involves the isolation of the compound and internal standard from plasma on a strong anion-exchange column at pH 10, followed by elution with 0.3 *M* acetic acid in methanol. The eluate is evaporated to dryness, and the residue is dissolved in dimethyl sulfoxide and permethylated with potassium *tert*-butoxide and methyl iodide. The permethylated compounds are reextracted from the reaction mixture with cyclohexane–methylene dichloride (9:1). The organic solution is evaporated to dryness, the residue is dissolved in ethyl acetate, and an aliquot is analyzed by GLC on a 3% OV-17 column. The extraction recovery from spiked plasma was 93.2 ± 2.1% (*SD*), whereas linearity for the overall procedure was in the 0–1-μg/ml range. The detection limit of the thermionic nitrogen detector was 50 ng/ml. The within-run and within-days precision (*CV*) were 4.0 and 6.2%, respectively, at 300 ng/ml.

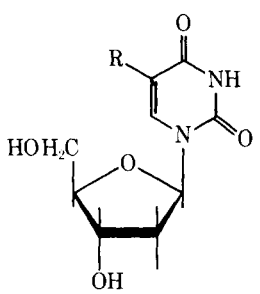
Keyphrases □ Floxuridine—GLC analysis in plasma □ GLC—analysis, floxuridine in plasma □ Antineoplastic–antiviral agents—floxuridine, GLC analysis in plasma

Biochemical studies (1) have shown floxuridine (5-fluoro-2′-deoxyuridine) (I) to be an intermediate in the formation of 5-fluoro-2′-deoxyuridine 5′-monophosphate, a potent inhibitor of thymidylate synthetase (2), a well-known target enzyme in cancer chemotherapy. This conversion takes place either directly by the action of deoxythymidine kinase (3) or indirectly by pathways in which I is first degraded to fluorouracil by nucleoside phosphorylase (4) and subsequently converted to 5-fluorouridine 5′-monophosphate (5, 6), which is finally metabolized to 5-fluoro-2′-deoxyuridine 5′-monophosphate by ribonucleotide reductase (7).

Cohen (8) identified I among the metabolites formed after administration of 1-(tetrahydrofuran-2-yl)-5-fluorouracil to rats. Several investigators (9–11) studied the physiological disposition of I and its metabolites after administration to patients. Therapeutic responses to this fluorinated nucleoside have been reported in various cancers (12–15).

Although several methods (16–22) were developed for fluorouracil analysis in biological fluids, few methods exist for the determination of I. Clarkson *et al.* (11) described a sensitive microbiological assay, which, however, is unable to differentiate between fluorouracil and I. Pantarotto *et al.* (18) published a mass fragmentographic method.

The present report describes a sensitive and simple GLC method for the measurement of I levels in human plasma; a thermionic nitrogen detector is used.



I: R = F
II: R = Cl

EXPERIMENTAL

Reagents—The potassium *tert*-butoxide[1] stock solution (6 g/100 ml) was prepared as reported previously (23) and diluted prior to use with an equal volume of dry dimethyl sulfoxide[2].

A carbonate buffer of pH 10 and ionic strength 0.1 was prepared by dissolving 2.1 g of sodium bicarbonate and 2.7 g of sodium carbonate in 1000 ml of double-distilled water.

The liquid scintillation cocktail consisted of 4 g of 2,5-diphenyloxazole[3], 50 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene[3], and 100 g of naphthalene[2]/liter of dioxane[2] (all scintillation grade).

All other inorganic and organic chemicals used were analytical grade, except methanol[2] (fluorometric quality).

Extraction Procedure—Pipet 1.0 ml of plasma, 100 μl of internal standard solution [2.1 mg of 5-chloro-2′-deoxyuridine[4] (II)/100 ml of distilled water], and 5.0 ml of carbonate buffer in a 15-ml centrifuge tube. Transfer the sample quantitatively (two subsequent washings with 1.0 ml of carbonate buffer) to a 2.0-cm × 6-mm i.d. glass column loosely packed with anion-exchange resin[5], plugged with glass wool, and fitted with a polytef tap and a 15-ml reservoir on top.

Allow the sample to pass through the resin bed and wash consecutively with 15 ml of distilled water and 15 ml of methanol; elute the compound and internal standard with 10 ml of 0.3 *M* acetic acid in methanol. Then evaporate the collected eluate to dryness in a water bath at 50° under a nitrogen stream.

Derivatization—Dissolve the residue of the column eluate in 200 μl of dry dimethyl sulfoxide. Add 50 μl of potassium *tert*-butoxide reagent, wait 10 sec, and then add 100 μl of methyl iodide. After 1 hr, add 5.0 ml of 0.05 *N* H_2SO_4; extract twice with 5.0 ml of cyclohexane–methylene dichloride (9:1). Evaporate the combined extracts to dryness in a heating block[6] at 50° under a nitrogen stream and dissolve the residue in 50 μl of ethyl acetate with the aid of a whirl mixer[7].

Parameters for GLC Analysis—A gas chromatograph[8] equipped with dual nitrogen–phosphorus thermionic detectors[9] was used for single-column operation. The stationary liquid phase, 3% OV-17[10], was coated on 100–120-mesh Gas Chrom Q[11] and packed into a 1.6-m × 2-mm i.d. spiral silanized glass column. The temperature settings were: oven, 205°; injection port, 250°; and detector, 300°.

Helium was used as a carrier gas at a linear velocity, $\bar{u}_0$, of 10 cm/sec; hydrogen and air flows were adjusted to 4 and 130 ml/min, respectively. The voltage controlling the temperature of the rubidium salt bead was set to obtain 30% of full-scale recorder deflection with an electrometer setting of 1 × 32, the zero control being turned fully counterclockwise.

The electrometer setting employed was 1 × 16. A 0–1-mv range recorder[12] was used. All injections were made on-column with a 10-μl sy-

[1] Fluka A. G., Buchs, Switzerland.
[2] E. Merck A. G., Darmstadt, West Germany.
[3] Packard Instrument Co., Downers Grove, Ill.
[4] Calbiochem A. G., Lucerne, Switzerland.
[5] AG 1-X4 (Cl^-), 100–200 mesh, Bio-Rad, Richmond, Va.
[6] Dri-Bath, Thermolyne, Dubuque, Iowa.
[7] Vortex Supermixer, Lab-Line Instruments, Melrose Park, Ill.
[8] Hewlett-Packard model 5710A.
[9] Hewlett-Packard model 18789A.
[10] Hewlett-Packard, Avondale, Pa.
[11] Supelco Inc., Bellefonte, Pa.
[12] Hewlett-Packard model 7123B.

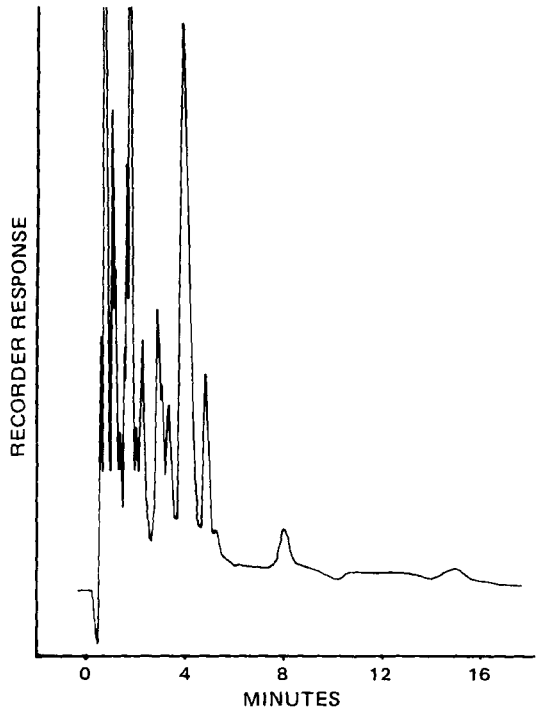


Figure 1—*Gas chromatogram of a drug-free plasma sample. The column was 3% OV-17 (oven temperature = 205°, $\bar{u}_0$ = 10 cm/sec).*

ringe[13], using an injection volume of 1 μl.

Linearity Experiments—Solutions of I[14] in citrate plasma of human origin were made containing 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 μg/ml. Samples (1.0 ml) were treated as described in the extraction and derivatization procedures and analyzed by GLC. Peak height ratios of I to the internal standard were plotted *versus* the concentration of I (micrograms per milliliter).

Recovery Experiments—To 1.0-ml plasma samples containing 1.0 μg of I/ml, 11 ng of 6-^{3}H-I[15] (specific activity of 2.3 Ci/mmole) was added. Dilutions with blank plasma were made to obtain 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 μg of I/ml (corresponding to 1.1, 2.2, 4.4, 6.6, 8.8, and 11 ng of 6-^{3}H-I/ml, respectively). Aliquots of 1.0 ml were then taken through the extraction procedure but without adding internal standard.

Column eluates were collected separately in polyethylene counting vials[3] and evaporated to dryness in a sand bath at 50° under a nitrogen stream. The residues were dissolved in 0.5 ml of distilled water, and 10 ml of liquid scintillation cocktail was added. Polyethylene vials containing 0.5-ml aliquots of original plasma samples and 10 ml of liquid scintillation cocktail were used as references to estimate extraction recoveries. All vials were counted for 5 min in a liquid scintillation counter[16]. The internal standard method, *i.e.*, adding 100 μl of tritiated water (specific activity of 0.94 μCi/g) to each counting vial, was used to correct for quenching.

Recovery values were calculated as follows:

$$\text{recovery (\%)} = \frac{\text{dpm of extract}}{(\text{dpm of reference sample}) \times 2} \times 100 \quad \text{(Eq. 1)}$$

RESULTS AND DISCUSSION

Due to its highly polar character, the I molecule is poorly extracted from aqueous solutions using double-phase extraction procedures. In addition, the required high polarity of the organic solvent system always causes many endogenous impurities to be coextracted from plasma, resulting in interfering peaks. Therefore, an on-column extraction technique (22) was developed.

Being a weak acid (pKa = 7.66) (24), I is present almost entirely as a monoanionic species (99.55%) at pH 10 and is quantitatively adsorbed on the anion-exchange resin employed. Other plasma constituents are removed with a water–methanol wash. Subsequent quantitative elution of I is performed with 0.3 *M* acetic acid in methanol. No apparent denaturation of plasma proteins occurs. The isolation of I from plasma was quantitative. Recovery experiments showed a mean of 93.2 ± 2.1% (*SD*) in the 0–1-μg/ml range.

The permethylation reaction was shown earlier (23) to yield stable low molecular weight permethylated nucleosides suitable for quantitative GLC. Initially, columns prepared with 3% OV-17[10], 3% SP-2250[11], 3% OV-25[10], and 1% STAP[11] (reaction product of Carbowax 20 M and succinic acid) were found to be superior in terms of selectivity and peak symmetry. Under the conditions described and with the 3% OV-17 column, the retention times, R_t, of I and the internal standard, 5-chloro-2′-deoxyuridine (II), were 6.4 and 12.0 min, respectively.

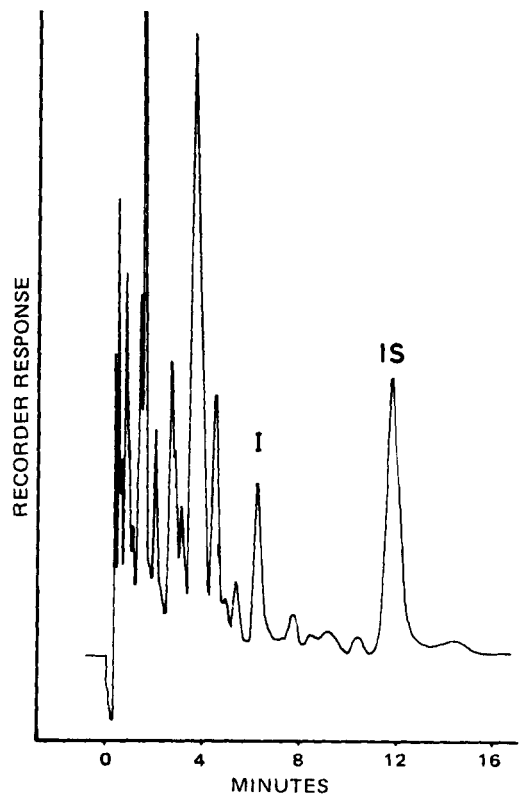


Figure 2—*Gas chromatogram of a plasma sample spiked with 500 ng of I/ml. The column was 3% OV-17 (oven temperature = 205°, $\bar{u}_0$ = 10 cm/sec). IS = internal standard.*

Table I—Precision of the I Assay

Long-Term Precision		12 Replicate
Day	ng/ml	Analyses, ng/ml
1	271	290
2	298	314
3	301	292
4	299	287
5	269	292
6	273	288
7	295	304
8	277	291
9	299	290
10	294	321
11	317	285
12	306	308
13	289	Mean 297
14	330	*SD* 12
15	299	*CV*, % 4.0
16	294	
17	268	
18	335	
19	285	
20	294	
	Mean 295	
	SD 18	
	CV, % 6.2	

[13] Hamilton 701, Reno, Nev.
[14] Sigma Chemical Co., St. Louis, Mo. (or Produits Roche N.V., Brussels, Belgium).
[15] Radiochemical Centre, Amersham, England.
[16] Tri-Carb 3390, Packard Instrument Co.

Compound II was chosen on the basis of its structural similarity to I. Due to its similar pKa (7.90) (25), II can be added directly to the plasma sample. Compound II also is easily and quantitatively permethylated with the method described. The derivative obtained is well extracted from the reaction mixture before GLC and yields excellent chromatographic characteristics on the 3% OV-17 column system. The chromatograms (Figs. 1 and 2) showed no major interfering peaks eluting in the regions corresponding to I and II.

The use of the thermionic nitrogen detector (26) provides higher sensitivity and better specificity for nitrogen-containing compounds. A linear relationship between peak height ratios of I to II and plasma concentrations of I was demonstrated in the 0–1-μg/ml range (regression line: $y = 1.02x - 0.01$, $r = 0.996$). A detection limit of 50 ng of I/ml was obtained by injecting 1.0 μl of the 50 μl of ethyl acetate from the reconstituted sample residues, using 1.0 ml of plasma for analysis.

The within-run precision of the overall procedure was determined by analyzing 12 plasma samples spiked with 300 ng of I/ml. A standard deviation and a coefficient of variation of 12 ng/ml and 4.0%, respectively, were found. Analysis of 20 plasma samples, also spiked with 300 ng of I/ml, over 3 weeks yielded some indication of the long-term precision, namely, 18 ng/ml (*SD*) and 6.2% (*CV*). These data on replicate analysis and long-term precision are presented in Table I and are acceptable for an analysis at the submicrogram level.

The described procedure is an example of the superiority of the thermionic nitrogen detector because of its selectivity for nitrogen-containing compounds. Although interference from other endogenous substances was expected, blank plasma samples and plasma samples spiked with I at the submicrogram level were taken through the procedure and gave no significant peaks that might interfere. Besides this increased selectivity, sensitivity is increased approximately 20 times compared to the flame-ionization detector.

The applicability of this method for plasma samples collected from patients treated with I was not demonstrated because I is not used as a chemotherapeutic agent to treat cancer cases in our medical environment. Since I occurs as a metabolite in humans treated with the antineoplastic agent fluorouracil, development of the described procedure is important. Therefore, we propose to extend our method for the assay of I in urine and tumorous tissue.

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ACKNOWLEDGMENTS

Supported by the National Medical Research Foundation (F.G.W.O.) through Grants 20452 and 3.004.76.

The authors are indebted to Produits Roche N.V., Brussels, Belgium, for their gift of I.

GLC Determination of Pemoline in Biological Fluids

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Received October 26, 1976, from the *Department of Pharmacy, University of Antwerp, Antwerp, Belgium.* Accepted for publication June 22, 1977.

Abstract □ A specific GLC method for the determination of microgram quantities of the central stimulant pemoline in biological fluids is described. Extraction problems due to the low solubility of pemoline are avoided by acid hydrolysis of the drug to 5-phenyl-2,4-oxazolidinedione, which then can be easily isolated by two-phase extraction with dichloromethane, ether, or chloroform. Amide–imide tautomerism enables a cleanup of the extract. Quantitative determination at the microgram level is done on a methylated fraction of the dichloromethane extract by GLC using a suitable internal standard. For supporting evidence of the GLC method's specificity, the compound is also identified by examining an aliquot of the final dichloromethane extract by TLC.

Keyphrases □ Pemoline—GLC analysis in biological fluids □ GLC—analysis, pemoline in biological fluids □ Stimulants, central—pemoline, GLC analysis in biological fluids

The synthesis of a series of oxazolidinones was reported (1), and later (2) the central stimulant activity of pemoline (2-imino-5-phenyl-4-oxazolidinone) was noted. Its pharmacology also was reviewed (3, 4).