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Short Communication

Determination of 2′-deoxy-5-iodouridine and its metabolite 5-iodouracil by high-performance liquid chromatography with ultraviolet absorbance detection in human serum

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ABSTRACT

A new assay is described for 2′-deoxy-5-iodouridine, a drug employed as an antiviral agent by topical application. The parent drug, its systemic metabolite 5-iodouracil and an internal standard (5-iodouridine) were extracted from salted serum by an ethyl acetate partition at pH 6.7, back-extracted in alkalinized water and injected into a reversed-phase column. Potassium phosphate buffer–acetonitrile (95:5, v/v) eluted the analytes at a flow-rate of 1.5 ml/min. Detection was at 290 nm. The method proved to be linear in the 100–2000 ng/ml range.

INTRODUCTION

Thymidine analogues such as 5-halogenated 2′-deoxyuridine are incorporated into the DNA of mammalian cells and increase their susceptibility to the lethal effect of X-irradiation [1,2]. This has led to the use of 5-bromodeoxyuridine as a therapeutic agent in the treatment of neoplastic disorders [3] and 5-iododeoxyuridine as an antiviral agent, against, for example, herpes simplex, herpes zoster and adenovirus [4,5], also using topical application [6]. The high-performance liquid chromatographic (HPLC) analytical evaluation of 5-bromodeoxyuridine and pyrimidines in biological fluids has resulted in three publications [7–9], and four papers deal with the assay of 5-iododeoxyuridine [4,10–12].

In the method of Simonetti *et al.* [4] the 2′-deoxy-5-iodouridine (IDU) peak,

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which overlaps the interfering endogenous one, reduces the selectivity and sensitivity.

In order to assay IDU and 5-iodouracil (IU) Klecker *et al.* [10] used two different analytical methods, while in our study they are measured simultaneously by a single procedure.

Saffhill and Hume [11] and Samuel *et al.* [12] separated [^{3}H]IDU or [^{125}I]IDU and its metabolite [^{3}H]IU or [^{125}I]IU in the plasma of animals *in vitro* using HPLC with a gradient elution system, counting the radioacitivity of the eluate using a beta or gamma counter, or HPLC and radioimmunoassay.

The above methods are not suitable for a bioavailability investigation in humans treated with topical IDU. This paper reports detailed procedures to assay IDU and its systemic metabolite (IU) in serum.

EXPERIMENTAL

Chemicals

Chemicals of analytical or HPLC grade were supplied by Carlo Erba (Milan, Italy) and Merck (Bracco, Milan, Italy). Working standards of IU, IDU and 5-iodouridine (IUR) (Fig. 1), the last used as an internal standard, were supplied by Sigma (Prodotti Gianni, Milan, Italy).

High-performance liquid chromatography

The HPLC unit included a Waters Assoc. (Milford, MA, USA) Model 600E system controller, a Waters Assoc. Model 481 UV detector, a Jasco (Tokyo, Japan) Model 851-AS autosampler and a Merck Hitachi (Tokyo, Japan) D-2000 Chromatographic integrator. The column used was a reversed-phase C_{18} column (Brownlee Labs., San José, CA, USA; 220 mm × 4.6 mm I.D., spherical, 5 μm

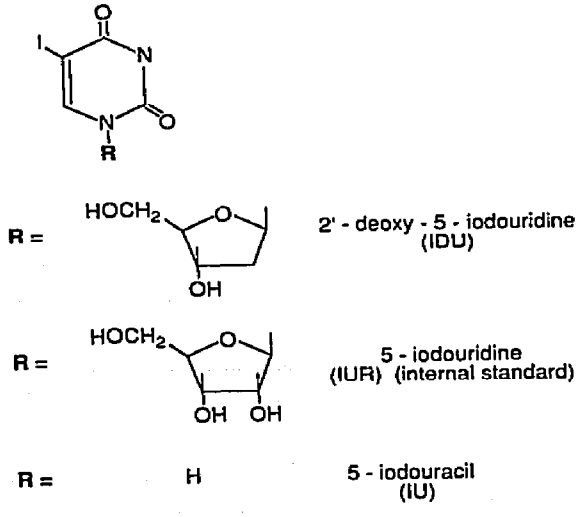

Fig. 1. Structures of substances involved in the investigation.

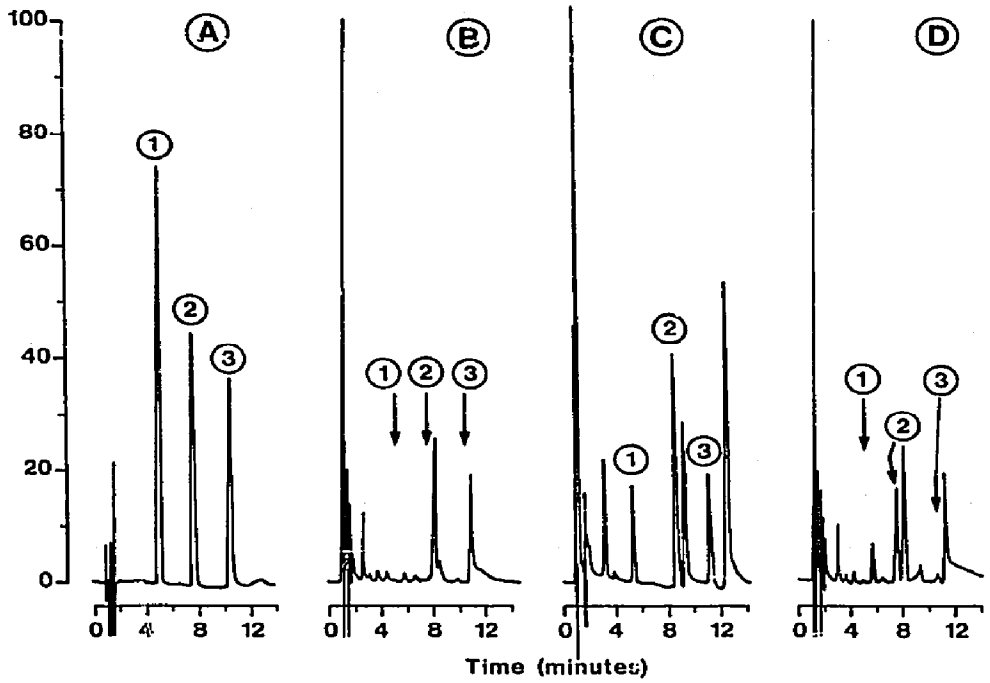

Fig. 2. Typical recordings of HPLC analysis. (A) Authentic standard solution of 5-iodouracil (IU), 5-iodouridine (IUR, internal standard) and 2'-deoxy-5-iodouridine (IDU). (B) Blank serum in the absence of analytes. (C) Blank serum spiked with the analytes (100 ng/ml). (D) A serum sample from one volunteer treated topically with IDU: neither IDU nor IU appeared; 500 ng of internal standard were added. Apart from the internal standard, other peaks are endogenous substances since they are also present in blank serum (B). Peaks: 1 = IU; 2 = IUR; 3 = IDU. HPLC conditions: column, Brownlee Labs. C_{18} (220 mm × 4.6 mm, I.D., spherical, 5 μm particle size); the eluent was a mixture of 0.01 *M* potassium phosphate buffer–acetonitrile (95:5); the flow-rate was set at 1.5 ml/min; detection was at 290 nm.

particle size). The eluent was a mixture of 0.01 *M* potassium phosphate buffer–acetonitrile (95:5). The flow-rate was set at 1.5 ml/min. Detection was at 290 nm. Under these conditions, the retention time of analytes was as follows: IU 5.15 min, IUR 7.74 min and IDU 10.33 min (Fig. 2).

Extraction from serum

A 0.5-ml sample of serum, 25 μl of internal standard solution (20 μg/ml IUR in eluent solution), 1 ml of saturated ammonium sulphate solution, 50 μl of ammonium phosphate buffered at pH 6.7 and 4 ml of ethyl acetate were pipetted into a 10-ml glass test tube. The tubes were tightly capped and vortex-mixed for 15 min at room temperature. After centrifugation at room temperature for 10 min at 2000 *g*, the ethyl acetate phase was transferred into a conical-bottomed glass tube and concentrated to approximately 0.5 ml by evaporation under a gentle stream of air. A 200-μl aliquot of 0.5 *M* sodium hydroxide was added to each sample, which was back-extracted by vortex-mixing for 15 min at room temperature. After centrifugation at room temperature at 2000 *g* for 10 min, the ethyl acetate phase was removed and discarded. A 50-μl aliquot of the alkaline aqueous phase was injected into the HPLC column.

TABLE I

EXTRACTION RECOVERY OF 2'-DEOXY-5-IODOURIDINE (IDU) AND 5-IODOURACIL (IU) FROM HUMAN SERUM

Concentration added (ng/ml)	IDU (n = 4)			IU (n = 4)		
	Recovered (mean ± S.D.) (ng/ml)	C.V. (%)	Recovery (%)	Recovered (mean ± S.D.) (ng/ml)	C.V. (%)	Recovery (%)
100	58 ± 9.0	15.5	58.2	53 ± 3.3	6.2	53.2
400	230 ± 22.2	9.7	57.5	194 ± 15.9	8.2	90.0
800	431 ± 38.9	9.0	53.9	378 ± 30.8	8.2	52.0
1000	615 ± 56.7	9.2	61.5	540 ± 37.5	7.0	47.2
1600	855 ± 66.7	7.8	53.4	758 ± 30.2	4.0	54.0
2000	1151 ± 97.0	8.4	57.5	949 ± 28.1	3.0	47.4
Mean			57.0			50.2
S.D.			3.0			3.2
C.V.			5.3			6.3

Clinical application

The whole investigation involved 12 healthy volunteers for checking systemic bioavailability, 24 healthy volunteers for tolerability trial, 40 patients suffering from herpes simplex and another 40 suffering from herpes zoster [13] in a double-blind controlled trial. Two gel formulations containing 10 and 40% IDU were administered to healthy volunteers; the 10% formulation was used in the herpes simplex trial and that at 40% concentration was administered in the herpes zoster investigation.

IDU and its systemic metabolite IU were assayed in timed serum samples from the above healthy volunteers and patients using the method described in this paper.

RESULTS AND DISCUSSION

The linearity of the method was assayed in the range 100–2000 ng/ml of serum for both IDU and IU. The linear regression was $y = 4.2119 + 0.5619x$ for IDU and $y = 14.5215 + 0.4722x$ for IU, the linear regression coefficient being in both cases 0.9999. The higher intercept encountered with IU is probably attributable to some endogenous interferences.

Extraction recovery from blank serum, investigated in the 100–2000 ng/ml range, was on average 57.0% with IDU and 50.2% with IU.

The intra-assay coefficient of variation (C.V.) proved to be in the range of 7.8–15.5% for IDU and 3.0–8.2% for IU. The inter-assay C.V. was 5.3% for IDU and 6.3% for IU (Table I).

When the internal standard was extracted from blank serum, a 40% average recovery was achieved.

The lowest detectable concentration in the case of IDU and IU proved to be 80 ng/ml, with C.V. of 18% for IDU and 8% for IU.

Fig. 2 shows typical recordings of (A) the analytes as authentic standards, (B) blank serum in absence of analytes, (C) blank serum spiked with the analytes (100 ng/ml) extracted and processed as described above and (D) a serum sample from one volunteer treated topically with IDU. The peak of IU appears at 5.15 min in an endogenous peak-free area. IUR, the internal standard, was eluted at 7.74 min, just before an endogenous peak; IDU was eluted at 10.33 min and was well separated from two endogenous peaks.

The literature on 5-bromodeoxyuridine assesses values in the range 40–50% as the best common recovery from deproteinized salted serum samples extracted with ethyl acetate in a liquid–liquid partition process [7,8], with a liquid–solid procedure [10] or with methanol cold-induced deproteinization [9]. From this viewpoint the 57% extraction recovery encountered here with IDU should be considered a useful finding in line with the literature on 5-halogenated 2'-deoxyuridine derivatives.

Several endogenous peaks were eluted with all the above methods, so skillful

handling is required to separate the peak of analytes from those produced by endogenous interferences. This has been fully achieved in the method described in this paper, allowing us to reach the highly satisfactory detection limit of 80 ng/ml, which is required to monitor the systemic absorption of IDU given topically.

Amine drugs, as are most hypnotics, sedatives, benzodiazepines, phenothiazines and histamine antagonists, do not interfere in the IDU assay because they are not extracted. Carboxylated non-steroidal anti-inflammatory agents could be marginally extracted but they do not interfere in IDU assay because they are not eluted. The method described in this paper is simple, possesses the validation required for pharmacokinetic and bioavailability purposes and needs only chemicals and apparatus commonly used in pharmacokinetic laboratories.

In the bioavailability investigation carried out on healthy volunteers topically treated with IDU, neither IDU nor its metabolite IU appeared in timed serum samples (Fig. 2D), leading us to conclude that IDU is not absorbed through the skin into the systemic circulation.

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