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Note

Determination of oxiracetam in human serum and urine by high-performance liquid chromatography*

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Oxiracetam (4-hydroxy-2-oxo-1-pyrrolidineacetamide, ISF 2522) (Fig. 1) is a new drug clinically effective in the treatment of cognitive disorders [1–3]. To investigate its basic pharmacokinetics in humans, a sensitive assay for human serum and urine is required.

The physicochemical properties of oxiracetam (30% water solubility, lack of suitable chromophores and degradation under gas chromatographic conditions) preclude its extraction and detection with an adequate sensitivity by high-performance liquid chromatography (HPLC) and selectivity by gas chromatography. The HPLC method described here analyses oxiracetam after derivatization to its triphenylsilyl ether.

EXPERIMENTAL

Reagents

Oxiracetam [1] ($C_6H_{10}N_2O_3$; mol. mass 158.16) and the internal standard [4] (ISF 2839, $C_7H_{12}N_2O_3$; mol. mass 172.18) (Fig. 1) were synthesized in ISF Labs. (Trezzano, Italy). Acetonitrile, dichloromethane, hexane, 2-propanol, methanol (HPLC grade) and pyridine (spectroscopic grade) were obtained from E. Merck (Darmstadt, F.R.G.). Citric acid (analytical grade) was obtained from Carlo Erba (Milan, Italy) and triphenylchlorosilane from Fluka (Buchs, Switzerland). Sep-Pak silica cartridges and the 0.5-μm FHLP filter used to filter all solvents before use were from Millipore (Bedford, MA, U.S.A.).

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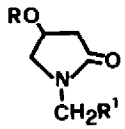

Fig.1. Structures of oxiracetam (ISF 2522) (**1**, R=H, $R^1=CONH_2$), internal standard (ISF 2839) (**2**, R=H, $R^1=CH_2CONH_2$), triphenylsilyl ether of **1** (**3**, $R=Si(C_6H_5)_3$, $R^1=CONH_2$) and triphenylsilyl ether of **2** (**4**, $R=Si(C_6H_5)_3$, $R^1=CH_2CONH_2$).

Chromatography

Chromatography was performed on a Hewlett-Packard Model 1080 B instrument equipped with a variable-wavelength UV detector (Perkin-Elmer LC-55) and Rheodyne (Berkeley, CA, U.S.A.) injector (50-μl loop). Detection was performed at 230 nm. The analytical column was a stainless-steel tube (250×4.5 mm I.D.) filled with 5-μm particles of silica (Bronwlee Labs., Santa Clara, CA, U.S.A.) and kept at 30°C. The mobile phase, hexane–2-propanol–water (77:22.5:0.5, v/v/v), was used at a flow-rate of 2 ml/min. Retention times were 6.30 min ($s=0.05$, $n=20$) for **3** and 9.30 min ($s=0.07$, $n=20$) for **4** (for structures see Fig. 1).

Sample preparation

*Serum.*To 0.5 ml of serum, 20 μl of an aqueous solution of internal standard (90 nmol, 15.5 μg) were added, and the mixture was freeze-dried in a freeze-drier (Modulyo, Edwards, Crawley, U.K.). Dry pyridine (1 ml) and triphenylchlorosilane (300 mg) were added to the residue, which was mixed and left at 60°C for 5 h to complete the reaction. After cooling, 8 ml of a saturated solution of citric acid (500 g/l) were added to the reaction mixture, which was extracted with 10 ml of dichloromethane for 1 min at 100 rpm on a horizontal mechanical shaker (Passoni, Milan, Italy) and centrifuged for 5 min at 2900 *g*. The aqueous phase was eliminated, and the organic layer was washed with 8 ml of the saturated solution of citric acid by shaking and centrifuging. The organic layer was filtered through a phase separator filter (1 PS, Whatman, Maidstone, U.K.) and evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 2 ml of dichloromethane and applied on a Sep-Pak cartridge by means of a 5-ml Luer-Lock syringe. [Before the application of the sample, the cartridge was rinsed with 10 ml of acetonitrile–water (8:2, v/v) and 10 ml of dichloromethane.] After application of the sample, the cartridge was rinsed with dichloromethane–acetonitrile (9:1, v/v). The eluate was collected in a test-tube and evaporated to dryness under vacuum at 50°C. The residue was reconstituted with 200 μl of acetonitrile, and 50 μl were injected into the chromatograph.

*Urine.*To 0.2 ml of urine, 20 μl of an aqueous solution of the internal standard (581 nmol, 100 μg) were added, and the mixture was freeze-dried. The procedure was the same as that described for serum, except that the amount of triphenylchlorosilane was reduced to 150 mg.

RESULTS AND DISCUSSION

Derivatization

The treatment of oxiracetam and of the internal standard with triphenylchlorosilane gave rise to the corresponding triphenylsilyl ethers. The parameters of the reaction (time, temperature, amount of reagents) were optimized on serum and urine blanks spiked with known amounts of oxiracetam.

Stability

Oxiracetam in serum and urine was stable when stored at −20°C for 30 days (no degradation occurred).

Calibration graphs

For serum, a calibration graph was established for the concentration range 9.67–252.91 nmol/ml (1.5–40 μg/ml) by adding equal volumes (20 μl) of aqueous solutions of oxiracetam at different concentrations to 0.5-ml aliquots of serum pool. To each sample, 15.5 μg of internal standard (ISF 2839) in 20 μl of water were added, and the mixture was then handled as described under *Sample preparation*. Peak-area ratios of the derivatives of oxiracetam and internal standard were calculated and plotted against the actual concentrations of oxiracetam.

For urine, concentrations were much higher, and a calibration graph was constructed in the range 0.19–9.17 μmol/ml (30–1450 μg/ml) as described for serum. Internal standard (100 μg) in 20 μl of water was added to each 0.2-ml sample of urine blank, and the samples were prepared as described. Peak-area ratios were calculated by dividing the area of the oxiracetam derivative peak by the area of the internal standard derivative signal and plotted against the actual concentration of oxiracetam.

The equations of the calibration curves were calculated by least-squares linear regression. The correlation coefficient of each curve was 0.9996.

Chromatographic selectivity

Preliminary metabolic studies in animals with labelled compounds [5] revealed that oxiracetam was excreted as such in urine and faeces without any evidence of metabolism. The chromatographic selectivity was thus checked for the naturally occurring endogenous compounds in serum and in urine. The chromatograms of serum and urine blanks and of serum and urine samples obtained from a subject after oral administration of 800 mg of oxiracetam (Figs. 2 and 3, respectively) revealed no interference.

Accuracy, precision and limit of quantitation

Accuracy, precision and limit of quantitation were calculated by analysing spiked serum and urine samples. Different concentrations, 3.37, 9.67, 64.47 and 156.80 nmol/ml (0.532, 1.529, 10.196 and 24.800 μg/ml) for serum and 190.0, 483.7 and 2569.5 nmol/ml (30.0, 76.5 and 406.4 μg/ml) for urine, were analysed on different days. The relative standard deviations (R.S.D.) and the deviation of the mean found from the given concentrations in serum and urine are listed in

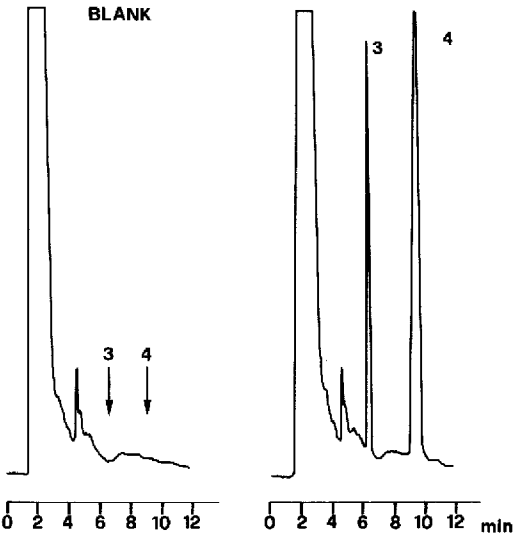

Fig. 2. Chromatograms obtained from a human serum blank (left) and from a serum sample (right) 12 h after oral administration of 800 mg of oxiracetam (oxiracetam found, 98.0 nmol/ml).

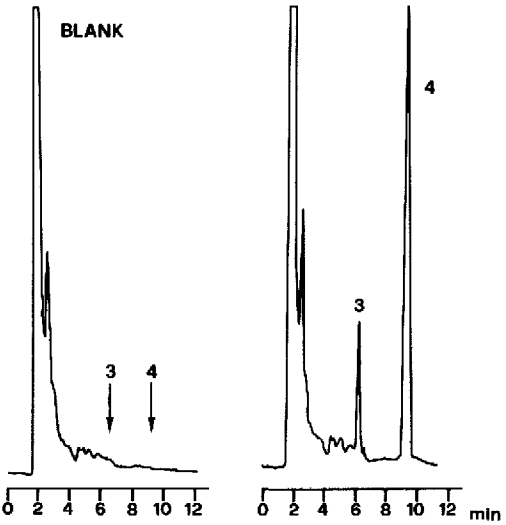

Fig. 3. Chromatograms of a human urine blank (left) and of a sample of urine (right) from a subject given 800 mg of oxiracetam orally (oxiracetam found, 483.7 nmol/ml).

Table I. The concentrations of 9.67 nmol/ml (R.S.D. 7.4%) for serum and of 190.0 nmol/ml (R.S.D. 8.7%) for urine were considered as the limits of quantitation; the concentration of 3.37 nmol/ml (R.S.D. 35.7%) was the limit of detection in serum.

Application

The applicability of the method for pharmacokinetic studies was tested by ana-

TABLE I

ACCURACY AND PRECISION FOR THE DETERMINATION OF OXIRACETAM IN HUMAN SERUM AND URINE

Amount spiked (nmol/ml)	Amount found (nmol/ml)	R.S.D. (%)	*n*	Deviation from theory (%)
Serum				
3.37	3.50	35.70	3	3.86
9.67	10.91	7.40	3	12.82
64.47	64.55	2.28	9	0.12
156.80	156.75	1.31	5	−0.03
Urine				
190.0	171.2	8.70	3	−9.89
483.7	470.1	5.66	4	−2.81
2569.5	2520.7	0.94	3	−1.90

lysing serum and urine samples from a subject given a single oral dose of 800 mg of oxiracetam in tablet form. Serum samples were collected over 12 h and urine samples over 24 h, and they were analysed for oxiracetam levels. The serum profile is shown in Fig. 4. The excretion of unchanged oxiracetam in urine was 71.7% within 24 h, in agreement with its bioavailability in animal species.

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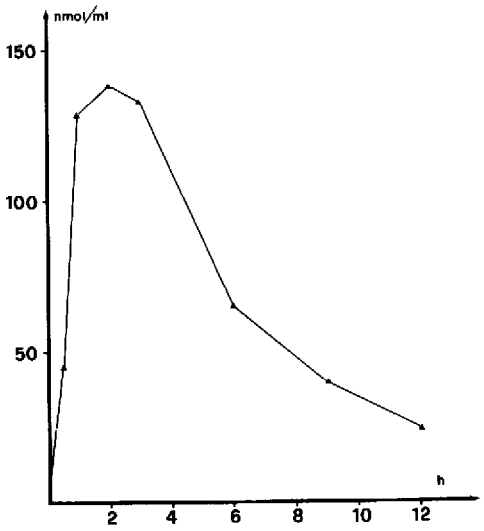

Fig. 4. Serum levels of oxiracetam (800 mg, single oral dose).

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