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DETERMINATION OF EXIFONE IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A high-performance liquid chromatographic method with electrochemical detection was developed for the determination of exifone in human plasma and urine Exifone was extracted from acidified plasma or neutralized urine with diethyl ether and the evaporated extracts were analysed on a C_{18} reversed-phase column The compound was eluted in about 8 min with acetonitrile–0 3 *M* orthophosphoric acid (15 85, v/v) at a flow-rate of 0 9 ml/min This method gave accurate and reproducible results, the calibration graphs were linear ($r > 0\ 99$) over the range of 2 8–360 nmol/l for plasma and 0 18–36 μmol/l for urine, and concentrations as low as 1 nmol/l in plasma could be quantified These results allowed this assay to be used for determinations in single-dose pharmacokinetic studies

INTRODUCTION

Exifone (Adlone®), hexahydro-2,3,4,3′,4′,5′-benzophenone (Fig. 1), has been proposed for the treatment of cognitive dysfunction in geriatric patients [1], Parkinsonian patients [2] and patients with memory defects [3]. Exifone possesses anti-radical properties that enable it to play a scavenger role [4].

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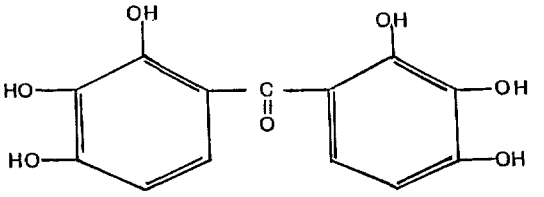

Fig 1 Structure of exifone

Methods for the determination of exifone in biological fluids are poorly documented; indeed, only one isotopic method with radiolabelled exifone followed by gas chromatography–mass spectrometry (GC–MS) [5] has been reported. GC–MS provides specific and sensitive results, but the constraints of cost and time involved in sample preparation and assay limit this method to special facilities and studies.

The purpose of this paper is to describe a simple, sensitive and selective assay for the determination of exifone in human plasma and urine by high-performance liquid chromatography (HPLC) coupled with dual-electrode coulometric detection. Owing to its six hydroxyl groups, exifone is a highly electroactive molecule. HPLC with electrochemical detection is the preferred method because it provides both selective and reproducible results combined with great sensitivity. The small sample volume (1 ml), the simple extraction and the low limits of detection (1 nmol/l) enable this assay to be used for single-dose pharmacokinetic studies in human subjects.

EXPERIMENTAL

Apparatus

Analyses were performed on an isocratic HPLC system consisting of a Model 712 WISP automatic injector (Waters Assoc., Milford, MA, U.S A.), a Model 590 pump (Waters Assoc.) and an ESA Model 5100 A coulometric detector (Environmental Sciences, Bedford, MA, U.S.A.) equipped with an ESA 5011 analytical cell. Chromatograms were traced on an Omniscribe strip-chart recorder (Houston Instruments, Houston, TX, U.S.A.). Chromatography was carried out on a 10 cm×0.5 cm I.D. Novapak C_{18} cartridge (Waters Assoc.) packed with 4 μm particle size spherical silica gel, with a Guard-Pack C_{18} (Waters Assoc.) precolumn. A radial compression module (RCM 8×10) (Waters Assoc.) was used to apply pressure to the cartridge.

Chemicals

Exifone in its yellow powder form (Batch No. 5R4567) was a gift from Pharmascience (Courbevoie, France). Acetonitrile (HPLC grade), orthophosphoric acid, toluene, citric acid, acetone and diethyl ether (analytical-reagent

grade) and concentrated phosphate buffer of pH 7 (Normadose) were obtained from Prolabo (Paris, France) Dimethyldichlorosilane was purchased from Fluka (Buchs, Switzerland). Distilled water was used for the preparation of all aqueous standards and buffered solutions

Chromatographic conditions

All separations were performed isocratically at room temperature (18–22°C) at a flow-rate of 0.9 ml/min An ESA Model 5011 dual-electrode cell was operated in the oxidative mode with electrochemical cell II voltage set at +0.30 V vs. an ESA reference electrode; the electrochemical cell I and the guard cell were not in use in this study. The gain of the detector was 10 μA to 10 nA full-scale. The mobile phase was acetonitrile–0.3 *M* orthophosphoric acid (15 85, v/v). The final pH was 2.2. This eluent was filtered through a 0.22-μm Millipore membrane and degassed under reduced pressure before use. Under these conditions, the retention time of exifone was 8 min.

Standard solutions

Exifone (100 mg) was dissolved in methanol and diluted to 100 ml in a volumetric flask. This stock solution was stored at +4°C for up to one month. Subsequent working dilutions were performed daily with 0.1 *M* citric acid to yield final concentrations over the range $1.8 \cdot 10^{-5}$–$3.6 \cdot 10^{-1}$ mmol/l.

Glassware silanization

Tubes were silanized by adding 0.5 ml of toluene–dimethyldichlorosilane (95 5, v/v). The tubes were capped and incubated for 12 h at 80°C. All the remaining glassware involved in the extraction procedure and in the preparation of the standard solutions was silanized by soaking in a toluene–dimethyldichlorosilane (95 5, v/v) bath for 12 h. Then two rinses with acetone were performed to remove the excess of the previous mixture

Human experiments

Volunteers received, after a 12-h fast, a 200-mg exifone tablet orally. Blood samples were taken from the forearm vein. Samples were drawn in lithium heparinized tubes at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12 and 24 h after drug administration Plasma was separated by centrifugation and samples were stored in plastic tubes at −20°C until analysed.

Extraction procedure

Plasma An aliquot (1 ml) of human plasma was transferred into a screw-capped tube (20 ml volume) and mixed with 900 μl of 0 1 *M* citric acid and 100 μl of the required dilution of exifone. After adding 8 ml of diethyl ether, the samples were extracted by agitation on a mechanical shaker for 10 min. Samples were centrifuged at 4°C for 5 min at 2400 *g*. The upper (7 ml) organic

layer was transferred into another 10-ml glass tube. The organic phase was evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted with 200 μl of mobile phase and automatically injected (20 μl) on to the HPLC column.

Urine The same procedure was employed for human urine samples except that citric acid was replaced with phosphate buffer (pH 7) The residue was reconstituted with 250 μl of mobile phase.

Calibration

Evaluation of the assay was carried out using nine standard points in the concentration range 2.8–360 nmol/l and 0 18–36 μmol/l for plasma and urine, respectively. The calibration graphs were obtained by linear regression of the peak height versus concentration. Recovery was calculated by comparing the measured values for spiked samples with those for standard aqueous solutions, titrating the same concentrations.

RESULTS

Linearity

The calibration graph for exifone in plasma was linear over the range 2 8–360 nmol/l and almost passed through the origin ($y=30\ 24x-1\ 62$; $r=0.9999$) The calibration graph for urine was linear over the range 0.18–36 μmol/l ($y=149x+0.8$; $r=0.9999$).

Recovery

The extraction recovery was determined by comparison between the detector response for plasma or urine samples spiked with exifone (36 nmol/l and 3.6 μmol/l, respectively) and the detector response of directly injected aqueous solutions. The absolute recoveries were 86% for plasma and 54% for urine.

Precision

To assess the intra-assay precision, coefficients of variation (C V) for the concentrations found versus concentrations added at four different levels (1, 7.2, 18 and 90 nmol/l) for plasma and three (0 7, 3.6 and 18 μmol/l) for urine were determined by running ten extracted samples of each concentration, as shown in Table I.

The inter-assay precision was determined by comparison of seven (plasma) and six (urine) concentrations in different runs obtained within a three-week period. The results are shown in Table II.

Limit of detection

Starting with 1 ml of plasma, the limit of detection was determined statistically by transposition of the average signal for ten drug-free plasma extracts

TABLE I

INTRA-ASSAY REPRODUCIBILITY AND ACCURACY OF EXIFONE DETERMINATION IN HUMAN PLASMA AND URINE SAMPLES

Concentration added	Concentration found (mean ± S D)	Coefficient of variation (%)	Accuracy (%)
Plasma (n = 10) (nmol/l)			
1	1 69 ± 0 10	9 0	155 0
7 2	6 71 ± 0 36	5 8	93 5
18	20 10 ± 1 29	6 6	112 0
90	97 56 ± 8 64	8 8	108 4
Urine (n = 10) (μmol/l)			
0 72	0 68 ± 0 25	4 7	95 0
3 6	3 81 ± 0 57	1 5	106 0
18	23 18 ± 1 08	4 7	128 8

TABLE II

INTER-ASSAY REPRODUCIBILITY AND ACCURACY OF EXIFONE DETERMINATION IN HUMAN PLASMA AND URINE SAMPLES

Concentration added	Concentration found (mean ± S D)	Coefficient of variation (%)	Accuracy (%)
Plasma (n = 6) (nmol/l)			
5 60	5 74 ± 1 75	8 9	102 5
11 20	10 62 ± 1 04	9 3	94 8
22 43	22 15 ± 3 26	15 0	98 7
44 87	43 72 ± 2 54	14 7	97 4
89 75	90 64 ± 4 41	3 1	101 1
179 50	176 62 ± 17 48	6 7	98 4
359 00	357 92 ± 6 64	6 5	99 7
Urine (n = 6) (μmol/l)			
0 90	0 88 ± 0 21	26 0	98 4
1 80	1 73 ± 0 25	15 0	96 4
3 59	3 34 ± 0 18	5 4	93 2
8 97	8 64 ± 0 17	2 4	96 4
17 90	18 40 ± 2 54	13 9	102 8
35 90	35 60 ± 1 18	3 3	99 3

on the linear regression curve. This theoretical limit was 0 81 nmol/l. The repeatability for ten plasma extracts previously spiked with 1 nmol/l exifone was established. Variance analysis and Student's *t*-test indicated that this limit of quantification is statistically significant. At this concentration (1 nmol/l) the coefficient of variation was 9%

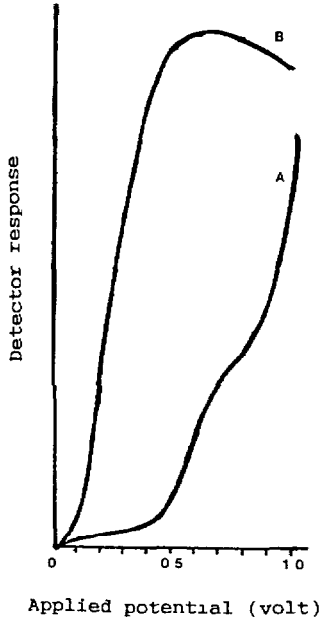

Fig 2 Hydrodynamic voltammograms (A) background current (mobile phase alone), (B) exifone in mobile phase after subtraction of the background current

Selectivity

The lower the detector potential chosen, the fewer were the interfering peaks The hydrodynamic voltammograms of exifone (Fig. 2) gave an optimum signal current when +0 50 V vs. an ESA reference electrode were applied to the electrode. However, such a potential was unworkable as it produced many interfering peaks and consequently the method would have suffered from poor selectivity As a compromise, we chose +0.30 V vs an ESA reference electrode as the detector potential in order to achieve better selectivity while maintaining sufficient sensitivity for the determination of the lowest levels of exifone found after oral administration

Typical chromatograms using the method described above are shown in Fig. 3.

Quality controls

During the assay and pharmacokinetic study, samples were spiked with a known amount of exifone and run blind simultaneously with biological samples. The concentrations found were compared with those expected and the results were analysed by linear regression ($y=0.9973x-0\ 85$; $r=0\ 9993$, $n=11$).

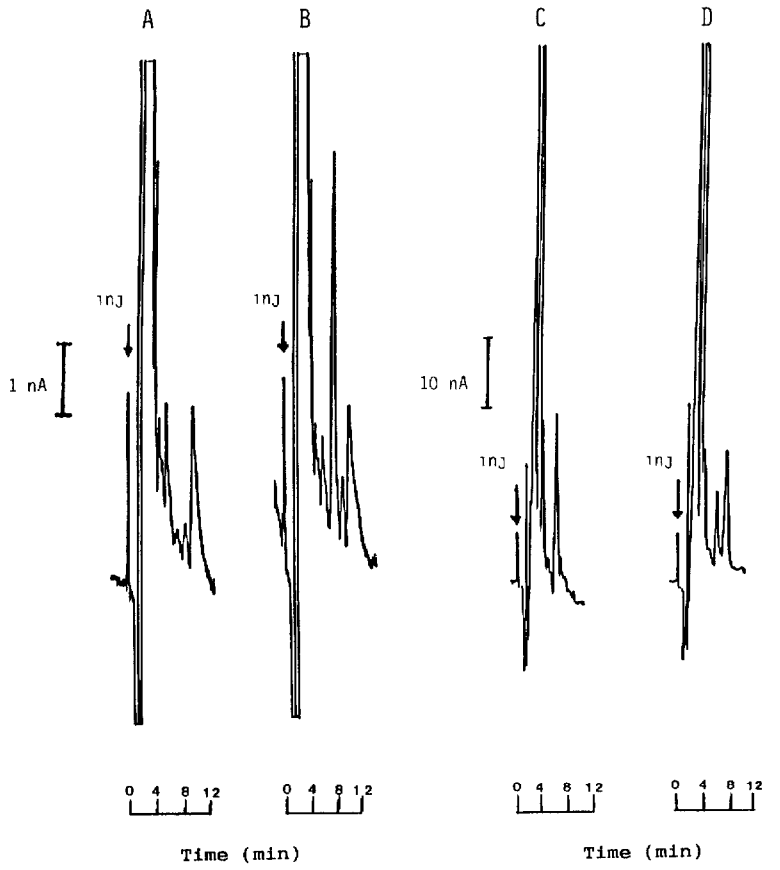

Fig 3 Chromatograms of (A) drug-free plasma, (B) drug-free plasma spiked with 1 8 nmol/l exifone, (C) drug-free urine and (D) urine spiked with 97 nmol/l exifone The C_{18} reversed-phase column was eluted isocratically with acetonitrile–0 3 *M* orthophosphoric acid (15 85, v/v) (pH 2 2)

Application of the method

An example of the plasma pharmacokinetic profile of exifone obtained after oral administration of a 200-mg tablet to one volunteer is presented in Fig. 4. The peak plasma concentration was observed 2 h after the oral administration of the drug with a C_{max} of 15 ng/ml. The terminal half-life calculated by linear regression on the last eight points on the terminal phase of the plasma concentration versus time curve was 18.6 h and the total area under the curve extrapolated to infinity by the linear trapezoid method was 18.6 μmol/l

DISCUSSION

Development of the method for the extraction of exifone from plasma and urine required preliminary characterization of the stability of the molecule in

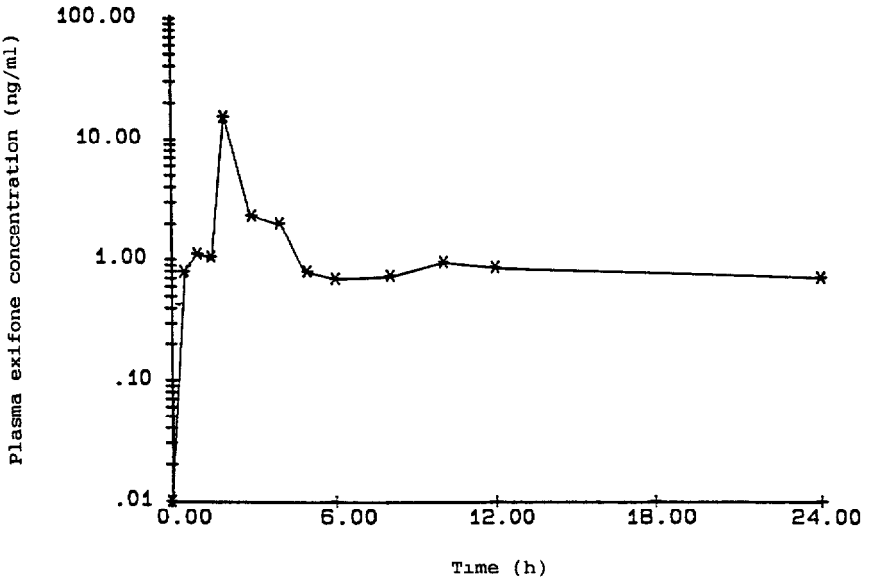

Fig 4 Plasma exifone concentration versus time curve after the oral administration of a 200-mg tablet

aqueous solutions as a function of pH. Exifone is rapidly transformed in alkaline solutions but this oxidation may be retarded in the presence of the antioxidant ascorbic acid. On the other hand, the molecule is stable in weakly acidic solutions such as boric, citric and orthophosphoric acids; in stronger acids, e.g., hydrochloric, nitric and sulphuric acids, exifone is unstable.

Extensive adsorption on glass but no detectable adsorption on plastic have been observed, and this problem was overcome by silanization of all the glassware.

Efforts to isolate and concentrate exifone from plasma and urine using liquid–liquid extraction with organic solvents indicated a better affinity for polar solvents. The recovery of exifone depends on the pH of the aqueous phase. The lower the pH, the better is the recovery but the greater the interferences. As a compromise, we chose pH 4 for plasma. On the other hand, such a clean-up procedure could not be applied to urine on account of the numerous endogenous products simultaneously extracted with exifone, and we were constrained to adopt pH 7 in spite of the poor recovery (54%). Nevertheless, the concentrations found in urine in a previous pharmacokinetic study [6] indicated that this method is sensitive enough.

The pH of the mobile phase had a considerable effect on retention time and sensitivity, as an increase in pH resulted in a delayed peak and a loss of sensitivity. Similar observations were made with variation of the mobile phase composition; moreover, electrochemical detection necessitates a mobile phase

of low ionic strength to provide adequate conductivity while minimizing contributions to the background current. A lower limit of detection might be obtained by increasing the injection volume and/or decreasing the volume of the reconstituted extract without impairing the selectivity of the method. Much of the improvement in this technique was obtained by the use of the radial compression module, which resulted in better selectivity, a shorter analysis time, a lower back-pressure and improved peak shape in comparison with conventional C_{18} or cyano reversed-phase columns.

In conclusion, an HPLC method with electrochemical detection has been developed for the determination of exifone in plasma and urine. The procedure is simple and rapid in comparison with other assays. The reproducibility and sensitivity of the method permit the accurate measurement of exifone to be achieved and allow pharmacokinetic investigations to be carried out

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