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ASSAY OF α-DIFLUOROMETHYLORNITHINE IN BODY FLUIDS AND TISSUES BY AUTOMATIC AMINO-ACID ANALYSIS

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SUMMARY

A procedure is described for the measurement of DL-α-difluoromethylornithine (DFMO), a selective irreversible inhibitor of ornithine decarboxylase, in biological specimens. The drug is separated from other amino acids with a commercial amino-acid analyser and detected by formation of its alkylthio-isoindole derivative with *o*-phthalaldehyde. DFMO concentrations of 0.1 nmol can be determined in a sample volume of 100 μl. The assay has been used to determine the half-life of DFMO in serum of several species and the relationships between serum and tissue concentrations.

INTRODUCTION

α-Difluoromethylornithine (RMI 71.782, DFMO) has been synthesized in this Centre to inhibit ornithine decarboxylase specifically and hence block production of putrescine and of the polyamines, spermidine and spermine [1]. The concentration of these three amines, which are essential to cell growth, is increased in rapidly proliferating tissues and they thus may be clinically relevant as biochemical markers of cancer [2]. DFMO has interesting properties when used in animal models of rapid cellular proliferation. Danzin et al. [3] have demonstrated that repeated doses of DFMO markedly slow the testosterone-induced weight gain of the prostate in castrated rats, while Prakash and co-workers have shown prolonged survival of L1210 leukemia-bearing mice [4] and retardation of the growth of solid EMT-6 mammary tumors in mice [5].

In order to carry out metabolic, pharmacological and distributional studies an assay for DFMO in biological samples was required. Procedures employing extraction and subsequent concentration of the drug were not applicable since DFMO is extremely water-soluble. Conventional automated amino-acid analysis using colorimetry with ninhydrin was tried initially. This was

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found, however, to be time-consuming and too insensitive for pharmacological studies. A method has therefore been developed where the chromatographic separation is made with a single buffer, so that only a certain section, or "window", of the amino acids measured in normal physiological assays is considered. By employing micro-columns and fluorimetric detection of the *o*-phthalaldehyde derivative, sensitivity has been increased so that 0.1 nmol of DFMO in 100 μl of an injected sample can easily be measured every 40 min.

METHODS

Chemicals

All materials were of A grade and were purchased from E. Merck (Darmstadt, G.F.R.) except the lithium citrate sample dilution buffer, which was obtained from Pierce (Rotterdam, The Netherlands). DFMO was synthesized in our centre [1].

Equipment

A Liquimat II (Kontron, Paris, France) with two 4 mm I.D. glass columns filled with DC-6a resin (Durrum, Palo Alto, CA, U.S.A.) to a bed height of 30 cm, was employed. The apparatus has the advantage that, while an analysis is in progress on one column, the other column is regenerated and then equilibrated in preparation for the second analysis. Two valves are switched automatically to ensure that the samples are injected on to alternate columns and that the effluent of the analysis column is directed to the fluorimeter. A two-column system is most efficient when short assay times are used since the proportion of time spent in regeneration and re-equilibration compared to analysis becomes significant using a single column.

Sample injection was made automatically by an APE-100 (Kontron) fitted with a 100-μl loop. Detection was carried out with the aid of a Fluoromonitor (American Instruments Co., Silver Spring, MD, U.S.A.). A flow cell of 70 μl was employed, with a Corning Glass 7-51 filter for the fluorescence excitation (340 nm) and a Wratten 2A for the emission (440 nm).

Analysis

Separation was made with a single eluting buffer (lithium citrate, 0.668 *M* Li^+, pH adjusted to 4.60 with HCl) using a flow-rate of 30 ml/h and column temperature of 54°C. Regeneration of the column was made after each analysis by passing lithium hydroxide (0.3 *M*) for 16 min. To form the fluorophore, the *o*-phthalaldehyde (OPA) reagent (17 ml/h) was mixed with the column eluent by means of a simple "T" connector and the reaction mixture passed immediately to the fluorimeter. The reagent consisted of 200 mg of OPA dissolved in 3 ml of methanol added to 1 l of 0.4 *M* (pH 10.4) potassium borate solution containing 3 ml of BRIJ and 1 ml of mercaptoethanol. The programme for the two columns is outlined in Table I. It shows an analysis time of 40 min for each column.

TABLE I

PROGRAMME FOR TWO-COLUMN ANALYSIS OF α-DIFLUOROMETHYLORNITHINE

Time (min)	Column 1	Column 2
0	Injection + eluting buffer	LiOH
16	Recorder and OPA* "on"	Re-equilibration
40	Analysis end, recorder and OPA "off"	Re-equilibration
40.01	LiOH	Injection + eluting buffer
56	Re-equilibration	Recorder and OPA "on"
80	Re-equilibration	Analysis end, recorder and OPA "off"
80.01	Repeat	Repeat

*OPA = *o*-phthalaldehyde.

Plasma extracts

Human or animal plasma and serum samples were diluted with one half volume of 20% trichloroacetic acid, allowed to stand for 30 min at 0°C to complete precipitation of the proteins, and then centrifuged for 2 min in an Eppendorf 3200 centrifuge.

The supernatant was further diluted with 0.2 *M* (pH 2.2) lithium citrate dilution buffer as required.

Urine

DFMO was excreted very rapidly in early urines collected after drug administration, so that a large dilution of urine samples could be made and injected directly on to the column without removing ammonia. Daily collections were made in polyethylene bottles, to which 1 ml of 6 *M* hydrochloric acid was added. An aliquot of 1 ml was then mixed with 250 μl of 20% trichloroacetic acid solution. After centrifugation 500 μl of the supernatant were further diluted to 10 ml with sample dilution buffer, before injection on to the column.

Organ extracts

DFMO has been measured in extracts of brain, liver, spleen, prostate and various tumours. Tissues were homogenised in 9 volumes of 0.2 *M* perchloric acid or 20% trichloroacetic acid and the supernatants were diluted and analysed.

Cell extracts

These were made in a similar fashion. Cells from rat hepatoma tissue culture (HTC) were centrifuged, washed with phosphate-buffered saline, and then sonicated with 0.9 ml of 0.1 *M* hydrochloric acid. Further dilution was then made by addition of 0.1 ml of 2 *M* perchloric acid.

Measurement

A 100-μl volume of sample extract was injected on to the column and the peak height of DFMO measured (retention time 30 min). The concentration

was calculated by reference to a calibration curve constructed from known standard solutions.

In the case of plasma samples, it was practical to prepare reference DFMO solutions in plasma, since slight differences in the slopes of the calibration curves obtained from plasma and aqueous samples were sometimes obtained (Fig. 1). Fluorescence was linear over the range 0.2—2 nmol per 100 μl injected.

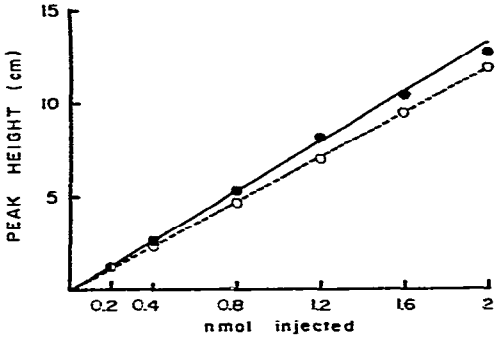


Fig. 1. Calibration plots of the peak height against the amount of DFMO injected. (•), DFMO in plasma; (○), DFMO in water.

Recovery

DFMO was added to plasma samples from different human volunteers to give a final concentration of 20 nmol/ml. They were then analysed by the procedure described for plasma samples. A recovery of 97.6% ± 2.9 (S.D.) (n = 6) was obtained. Similar recoveries were obtained when various tissue extracts were carried through the procedure. No significant change in the DFMO content of samples has been observed after storage at —20° C for several months.

RESULTS AND DISCUSSION

Fluorophore formation

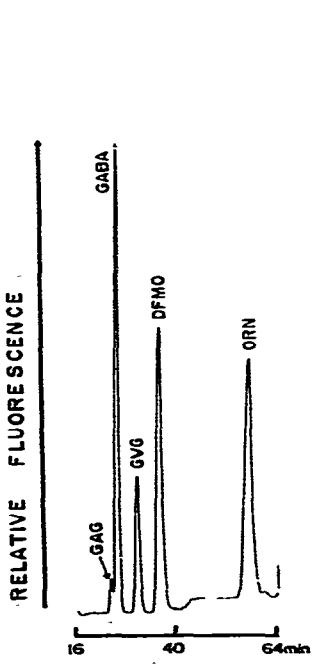
The reaction of *o*-phthalaldehyde and primary amines in the presence of mercaptoethanol was first reported by Roth [6] and subsequently applied to automated amino-acid analysis [7]. The identity of the fluorophores has been established recently by Stoney Simons and Johnson [8] as 1-alkythio-2-alkyl-isoindoles. It seemed evident that this reaction might be applied to α-difluoromethylornithine, and indeed the fluorescence quantum yield obtained was similar to that of ornithine. As mentioned above the mixing of column effluent and OPA solution was carried out without temperature control and the tube length (i.e. reaction time) between the mixing "T" and the fluorimeter was kept to a minimum, since this gave optimum response for the fluorescence of DFMO. Analysis was always made within the range of the calibration curve, i.e. 0.2—2 nmol per 100 μl injected, although a linear relative fluorescence/concentration relationship was found up to 10 nmoles

injected. Special precautions were not found to be necessary to avoid degradation of the OPA reagent if the volume prepared was used up within 2—3 days.

Chromatography

Short programmes for the separation of a few selected amino acids have been recently discussed by Olek et al. [9]. By eluting with a single buffer only a certain portion or "window" of the amino acids normally encountered in a physiological analysis are measured. The choice of the eluting buffer in the present assay was determined by our interest in inhibitors of both γ-aminobutyric acid (GABA) and ornithine metabolism. Fortunately, DFMO elutes between these two acids (Fig. 2), and indeed two GABA transaminase inhibitors, γ-acetylenic GABA (RMI 71.645) and γ-vinyl GABA (RMI 71.754), can also be separated with the present method. It is probable, therefore, that this assay can be used in studies involving these other inhibitors.

Fig. 3 shows chromatograms of human plasma carried through the procedure from a volunteer before and 4 h after ingestion of 10 mg/kg DFMO. Similar chromatograms have been obtained with plasma, or sera, from mouse, rat, rabbit, monkey and dog. Even haemolysed samples and extracts from sonicated



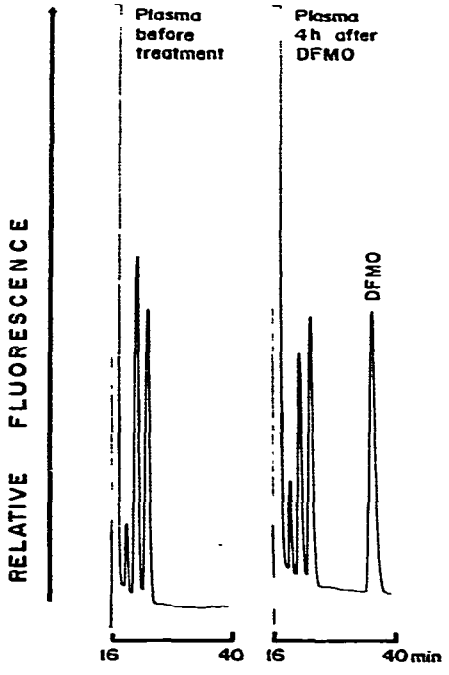


Fig. 2. Separation of γ-acetylenic GABA (GAG), GABA, γ-vinyl GABA (GVG), DFMO and ornithine (ORN) using the lithium citrate buffer for elution and OPA fluorophore formation for detection, as described in the Methods section, but using a longer elution time. One nanomole of each acid was injected.

Fig. 3. Chromatograms of human plasma prepared as described in the text. Left: blank plasma, i.e. before administering DFMO. Right: plasma sample from the same individual 4 h after taking DFMO 10 mg/kg orally.

red blood cells give "clean" chromatograms. The same remarks are applicable to all organ extracts so far analysed.

Fig. 4 shows a chromatogram of aliquots of human urine from samples collected for 24 h before and after ingestion of 10 mg/kg DFMO. Again no interference is evident, although if the drug concentration is low, as in later urine collections, some minor peaks can occur when a smaller dilution is made.

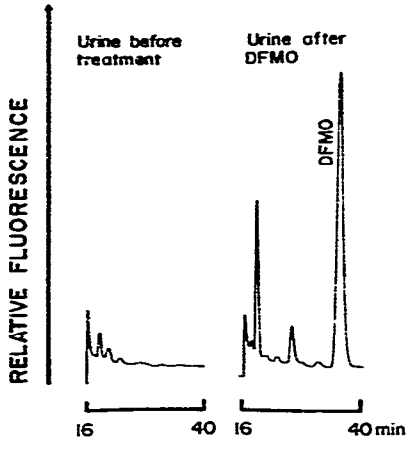


Fig. 4. Chromatograms of human urine prepared as described in the text. Left: blank urine from a 24-h sample before taking DFMO. Right: urine sample of the same individual from a 24-h collection after taking DFMO 10 mg/kg.

Application to biological samples

The assay has been used in pharmacological studies, particularly in a comparison of plasma half-lives of the drug in various species. It is sufficiently sensitive to enable the drug concentration profile to be followed even in individual rats. A catheter inserted in the rat femoral artery enabled blood samples (200 μl) to be taken at intervals for measurement of DFMO concentration. The drug could be measured for 6 h after giving the rats an oral dose of 200 mg/kg DFMO (Fig. 5A) and for 20 h in the monkey after 10 mg/kg orally (Fig. 5B). A comparison of the plasma half-life and urinary excretion of DFMO in various species is given in Table II.

It has also been important in several studies to demonstrate the presence of DFMO in the target organ. DFMO has been shown to enter the principal organs, to cross the blood—brain barrier and to enter tumours whose growth it retards [5]. The concentration of DFMO responsible for different biological effects in various animal models has been compared. For example, the concentration of DFMO that reduced the appearance of new tumours in rats treated with dimethylbenzanthracene and that exhibited contragestational effects in several species was around 10^{-4} *M*.

Similarly, in an evaluation of DFMO using cell-culture preparations, its ability to inhibit the target enzyme ornithine decarboxylase and consequently to affect polyamine biosynthesis has been compared to other inhibitors. In this respect, the inhibition of ornithine decarboxylase depends on the facility with which the inhibitor enters the cell. Investigations of the extra-

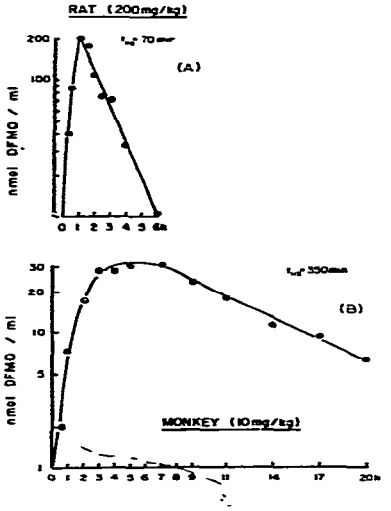


Fig. 5. Comparison of the plasma concentrations of DFMO in (A) rat after oral administration of 200 mg/kg, and (B) monkey after an oral dose of 10 mg/kg.

TABLE II

PLASMA HALF-LIFE OF DFMO AND ITS URINARY EXCRETION BY VARIOUS SPECIES

Species	Oral dose (mg/kg)	Half-life (min ± S.D.)	Amount of DFMO excreted in urine (0—24 h) (% ± S.D.)
Cynomologus monkey (n = 3)	10	353 ± 55	22.2 ± 3.5
Beagle (n = 2)	10	108, 132	26.9, 57.9
New Zealand white rabbit (n = 3)	10	121 ± 30	—
Sprague—Dawley rat (n = 4)	200	83 ± 30	82.5 ± 5.0*

*In the rat < 1% was excreted during 24—48 h.

and intra-cellular distribution of DFMO as a function of the medium concentration have therefore been made. Fig. 6 shows (A) the amount of DFMO found in the cells as a function of volume of cell culture taken for analysis with different concentrations of DFMO in the medium, and (B) the linear correlation between intra-/extra-cellular concentration of DFMO. The results indicate that there is passive diffusion of DFMO.

In summary, the method described has been used continuously for over a year for the measurement of DFMO in a variety of tissue extracts and biological fluids from several species. Interfering products which might invalidate the assay have never been encountered, even when DFMO concentrations were in the sub-nanomole range.

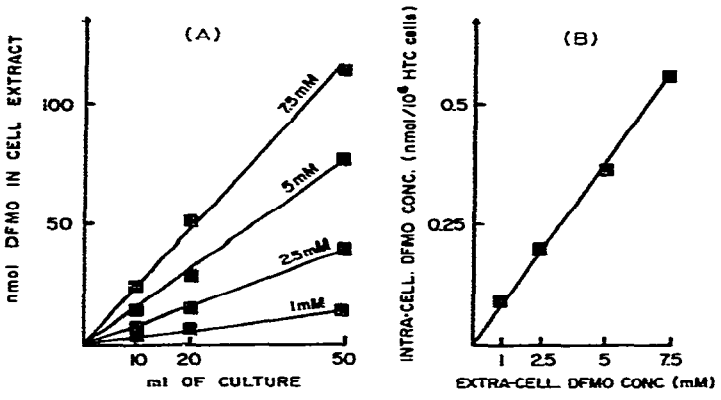


Fig. 6. Intracellular incorporation of DFMO by cultured rat hepatoma cells. (A) Amount of DFMO found within the cells from different volumes of culture with various extracellular DFMO concentrations in the medium. Number of cells per ml of culture was $3 \cdot 10^5$. (B) Relationship of intra- and extra-cellular DFMO content of cultured rat hepatoma cells after 48 h incubation.

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