Journal of Chromatography, 276 (1983) 197—201
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1725

Note

Measurement of 2-amino-N-(1,1-dimethylhexyl)acetamide (A643C), an investigative antidepressant, in plasma by electron-capture gas chromatography

D.N. PARSONS

Department of Drug Metabolism, Wellcome Research Laboratories, Beckenham, Kent BS3 3BS (Great Britain)

(First received January 7th, 1983; revised manuscript received March 7th, 1983)

2-Amino-N-(1,1-dimethylhexyl)acetamide (A643C; structure I, Fig. 1) has a pharmacological profile in animal models indicative of a potential antidepressant. To allow the investigation of the pharmacokinetics of this compound in laboratory animal species and man, a selective and sensitive analytical method was necessary. This report describes conditions for submicrogram derivatisation of A643C from plasma with pentafluoropropionic anhydride and for determination of the resulting pentafluoropropionamide by electron-capture gas chromatography.

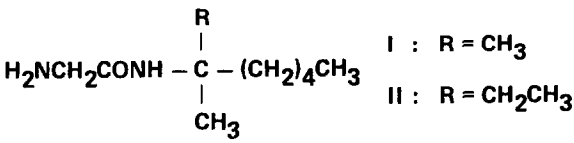

Fig. 1. Structures of A643C (I) and A404C (II).

MATERIALS AND METHODS

Reagents and materials

A643C hydrogen succinate and the homologue A404C hydrogen oxalate (2-amino-N-(1-methyl-1-ethylhexyl)acetamide; structure II, Fig. 1) used as internal standard were synthesised at the Chemical Research Laboratories, Wellcome Research Laboratories (Beckenham, Great Britain).

Other chemicals used were: pentafluoropropionic anhydride (PFPA; Pierce and Warriner, Chester Great Britain); *n*-hexane (Rathburn Chemicals, Walkerburn, Great Britain); OV-225 (Phase Separations, Queensferry, Great Britain);

0378-4347/83/$03.00

and Chromosorb W HP 100—120 mesh (Analabs, North Haven, CT, U.S.A.). All other chemicals and reagents were analytical grade and were obtained from BDH (Poole, Great Britain). Plasma used for the development of the assay was obtained from blood taken by venipuncture from healthy volunteers who had not received any drug treatment for at least one month before blood withdrawal.

Glassware

Screw-capped 20-ml glass tubes were used for the extraction and solvent evaporation.

Instrumentation

A Perkin-Elmer F30 instrument, modified to allow sample injection from a Hewlett-Packard 7670A autosampler, was used with a ^{63}Ni electron-capture detector. Operation of the autosampler and processing of data were carried out using a Hewlett-Packard 3352B minicomputer-based laboratory automation system.

Extraction procedure

A643C and A404C were prepared as stock solutions of their respective salts in water. They were prepared weekly and stored at 4°C. Depending upon the concentration of A643C expected and the volume of sample available, replicate 0.1—1 ml portions of plasma were taken for extraction. A404C was added (250 ng in 0.1 ml) followed by 1 ml of 4 *M* sodium hydroxide. The mixtures were extracted on a tumbler for 15 min (15 rpm) with 10 ml *n*-hexane. After centrifugation at 2000 *g* for 10 min, the organic layers were transferred to clean tubes. A 2-ml volume of 2 *M* hydrochloric acid was added and the tubes shaken vigorously on a mechanical shaker at approximately 275 strokes min^{-1} for 15 min. The phases were separated by centrifugation at 2000 *g* for 10 min and the supernatants removed by aspiration and discarded. The aqueous layers were made basic by the addition of 0.8 ml of 10 *M* sodium hydroxide, 10 ml *n*-hexane were added and the mixture extracted on a box tumbler for 10 min. The phases were separated by centrifugation at 2000 *g* for 10 min and the supernatants transferred to clean tubes. PFPA (30 μl) was added and the tubes placed on a box tumbler for 15 min. Excess reagent and solvent were removed under a stream of nitrogen gas at room temperature. Care was taken to remove tubes as soon as they were dry. The residues were redissolved in 0.4 ml cyclohexane, using a Vortex mixer to wash the sides of the tubes. Samples were transferred to Hewlett-Packard microvials for analysis by gas chromatography; sample injection volume was 5 μl.

Gas chromatography

After preliminary evaluation of a number of stationary phases, it was concluded that 5% OV-225 on Chromosorb W HP (100—120 mesh) in a 4 m × 3 mm I.D. glass column was optimal in terms of separating the drug and internal standard from extraneous biological material. The injection port of the gas chromatograph was maintained at 225°C, the detector at 350°C and the column at 210°C. Argon was used as carrier gas at a flow-rate of 40 ml min^{-1}.

Quantitation and calibration

The Hewlett-Packard 3352B data system identified A643C and A404C by retention time, measured their peak areas and calculated the peak area ratios. Calibration curves were constructed by a weighted linear regression of peak area ratios against the concentrations added. A weighting factor of 1/(concentration)^2 was used. Concentrations of unknowns were calculated from peak area ratios by interpolation of the computed calibration curve. Calibration curves were linear with zero intercepts. Precision and accuracy were essentially constant with a relative standard deviation below 3.5% over the range 10—250 ng ml^{-1} (Table I).

TABLE I

PRECISION AND ACCURACY OF A643C ANALYSIS IN DOG PLASMA

Known value (ng ml^{-1})	Assayed value (ng ml^{-1})	Standard deviation (n = 6)	Relative standard deviation (%)
10	10.1	0.3	2.6
50	48.7	1.5	3.2
100	101.0	3.4	3.4
250	254.1	2.6	1.0

RESULTS AND DISCUSSION

A typical gas chromatogram of a derivatised extract from dog plasma is shown in Fig. 2. Studies with radiolabelled A643C show that the overall extraction efficiency is 61 ± 3% (n = 6). Although not giving the highest extraction efficiency of the range of solvents initially investigated, hexane was chosen as extracting solvent because it gave an efficiency which was more than adequate for the levels of A643C encountered and because the resultant chromatograms were free from any interfering peaks. The minimum detectable quantity of pure drug (signal-to-noise ratio = 2) is 100 pg injected on column. The lowest measurable concentration in plasma is approximately 10 ng ml^{-1}.

In addition to problems with adsorption onto glassware used in pre-chromatographic manipulations, the analysis of free amines by gas chromatography is frequently unsatisfactory due to peak tailing and low sensitivity caused by adsorption of the sample onto the chromatographic support. In most cases derivatisation of the amine is necessary to achieve optimal chromatographic characteristics. At a very early stage in the development of an assay for A643C it became apparent that, whilst there were no problems with adsorption onto glassware, the formation of a suitable derivative would be essential both to improve chromatography and to provide adequate sensitivity. Perfluoroalkyl anhydrides are commonly used for the derivatisation of amines and amides [1, 2]; for example trifluoroacetic (TFAA) [1, 3, 4] pentafluoropropionic (PFPA) [5, 6] and heptafluorobutyric (HFBA) [7—9] anhydrides. Preliminary experiments showed the TFAA and HFBA derivatives of A643C to be unsuitable because of instability and excess reagent removal difficulties,

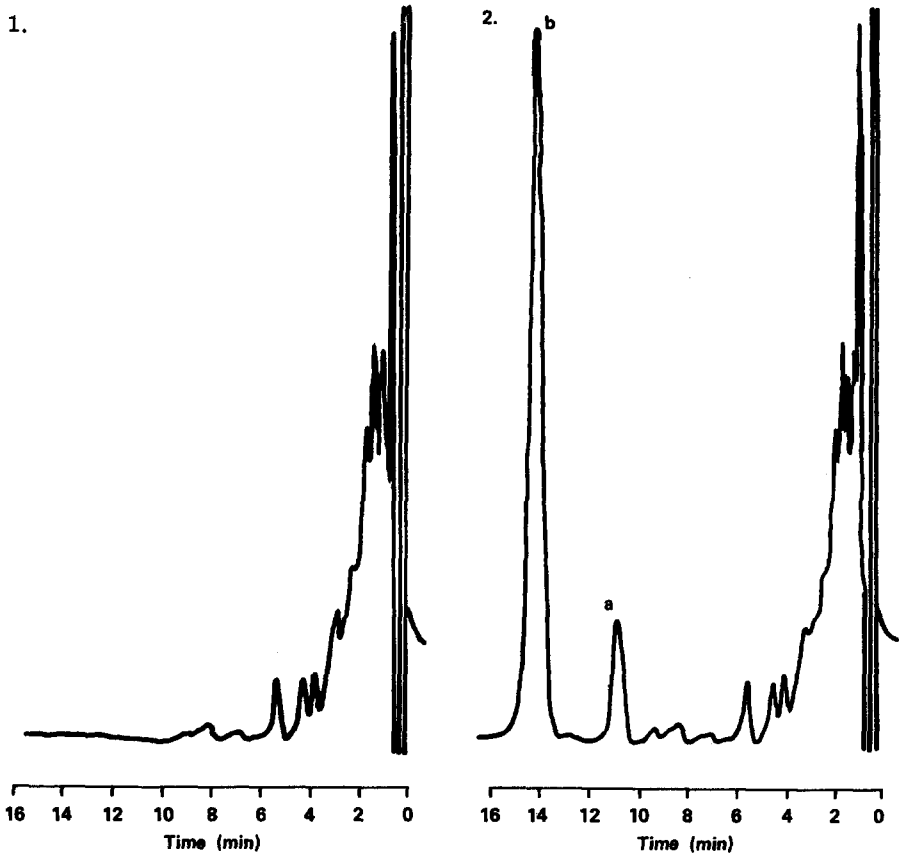

Fig. 2. Gas chromatograms of dog plasma samples extracted and analysed for A643C. (1) Control plasma; (2) plasma containing 50 and 250 ng ml^{-1} of A643C and A404C, respectively. Peaks: a = A643C pentafluoropropionamide; b = A404C pentafluoropropionamide.

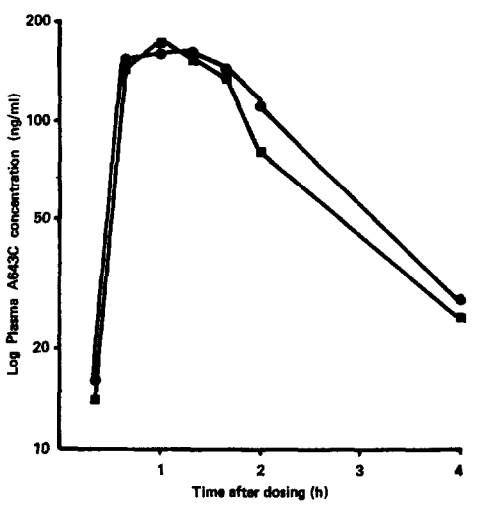

Fig. 3. Plasma concentration—time profile of A643C in two male beagle dogs dosed orally with A643C hydrogen succinate at 10 mg kg^{-1}.

respectively. No attempts were made to overcome the reagent removal problems with HFBA as an additional step in the assay procedure was undesirable. Although perfluoroamide derivatives of amines are frequently sensitive to hydrolysis [1, 2, 10] the stability of the pentafluoropropionamides of A643C and A404C is very good. A decrease in peak area ratios is noticed after two weeks. After three days storage at 4°C a decrease in peak areas of only a few percentage units is noted but with peak area ratios remaining constant. As a routine, samples are analysed within two days of extraction and derivatisation. The improved stability of a PFPA derivative over that of the corresponding TFAA derivative has been previously reported [7, 10].

The reaction of A643C and A404C with PFPA at room temperature is rapid and results in a quantitative conversion of the primary amines to their respective monopentafluoropropionamide derivative. The identities of these derivatives were established by gas chromatography—mass spectrometry. Reactions at elevated temperatures (60°C+) resulted in a non-reproducible mixture of di- and monopentafluoropropionamides. Three derivatives of each compound were identified: the mono-amine and amide derivatives and the di-amine/amide derivatives.

The method described is selective, sensitive and reproducible. Although described for dog plasma, it can also be applied to the analysis of A643C in human plasma and urine with no modifications. The suitability and application of this method to pharmacokinetic studies have been established (Fig. 3) and will be reported elsewhere.

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