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DETERMINATION OF PLASMA AND URINARY LEVELS OF AN ANALGESIC ANTI-INFLAMMATORY α-[4-(2-ISOINDOLINYL)PHENYL]PROPIONIC ACID DERIVATIVE BY GAS–LIQUID CHROMATOGRAPHY

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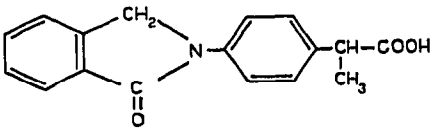
SUMMARY

A method for the gas–liquid chromatographic determination of *dl*-α-[4-(1-oxo-2-isoindolinyl)phenyl]propionic acid in human plasma and urine is described. The substance is extracted from acidified plasma or urine with added internal standard, purified by partition and submitted to gas–liquid chromatography after conversion to its 2,2,2-trifluoroethyl ester. Data on the recovery reproducibility and sensitivity of the method are given. Plasma levels and urinary excretion over time in a subject treated by the oral route with 100 mg of the product are presented.

INTRODUCTION

In the course of investigations on the analgesic and anti-inflammatory activity of isoindoline derivatives[1], *dl*-α-[4-(1-oxo-2-isoindolinyl)phenyl]propionic acid (see below) proved particularly promising[2]. Metabolic studies carried out in man using



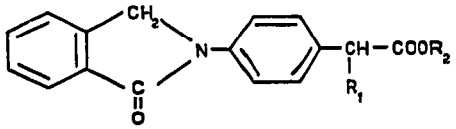
the same substance labelled with ^{14}C have shown that most of it is present unchanged in plasma and as the glucuronide in urine[3]. In order to determine the parameters describing the pharmacokinetics and bioavailability of the substance in man, a specific method was required to measure its plasma and urinary levels above 0.3 and 5.0 μg/ml, respectively.

MATERIALS AND METHODS

Table I reports the structural formulae, the physical characteristics and abbreviations used to designate each reference compound in this work. Compounds I, II and III were synthesized by methods described in the literature[1]: their titre (potentiometric titration with alcoholic NaOH) was 100% for I and II and 99.8% for III. Compounds I-tfe, II-tfe and III-tfe were synthesized by reacting I, II and III

TABLE I

PHYSICAL CHARACTERISTICS OF ISOINDOLINYLPHENYLPROPIONIC ACIDS AND THEIR DERIVATIVES



R_1	R_2	Abbreviation	Melting point (°C)	R_F *	Retention time (min) **
CH_3	H	I	213–214	0.41	
C_3H_7	H	II	160–161	0.50	
C_4H_9	H	III	144–146	0.49	
CH_3	CH_2CF_3	I-tfe	121–122	0.70	3.42
C_3H_7	CH_2CF_3	II-tfe	110–112	0.71	5.00
C_4H_9	CH_2CF_3	III-tfe	107–109	0.64	6.45

* On silica gel thin layers with chloroform–methanol–water (170:30:2) as solvent system.
** For GLC conditions see text.

with BF_3–2,2,2-trifluoroethanol, as described in the following paragraph. 2,2,2-Trifluoroethanol (Pierce, Rockford, Ill., U.S.A.) was used without further purification. BF_3 was obtained from J. T. Baker (Phillipsburgh, N. J., U.S.A.). BF_3–2,2,2-trifluoroethanol 30% (w/v) was prepared by bubbling BF_3 slowly through 2,2,2-trifluoroethanol at 0°; this reagent showed no change in its esterification capacity after one month at 0°. All other solvents and reagents were from Carlo Erba (Milan, Italy).

Synthesis of I-tfe, II-tfe and III-tfe

Preliminary work had shown that the derivatives of the carboxylic acids I, II and III best suited for their GLC determination in plasma and urine extracts were the 2,2,2-trifluoroethyl esters. These derivatives were easily prepared in quantitative yield by reacting the carboxylic acids with BF_3–2,2,2-trifluoroethanol using the following procedure. BF_3–2,2,2-trifluoroethanol (5 ml) was added to 500 mg of I, II and III and the suspension was heated at 60° until the substance was completely dissolved (about 15 min). The excess reagent was evaporated under vacuum and by addition of ethanol a crystalline product was obtained from the residual oil, and this was further crystallized from ethanol. Elemental analysis data for each 2,2,2-trifluoroethyl ester were in accordance with those required by the empirical formulae (I-tfe = $C_{19}H_{15}F_3NO_3$, II-tfe = $C_{21}H_{20}F_3NO_3$, III-tfe = $C_{22}H_{22}F_3NO_3$). The structure of these compounds was confirmed by the presence of some characteristic bands in their infrared (IR) spectra. In particular the IR spectra (in CCl_4) of these esters showed bands at 1750–1755 cm^{-1}, 1704–1705 cm^{-1} and 1168 cm^{-1}, attributable to ν (CO–O), ν (CO–N) and ν (CF_3), respectively. In addition, when examined as crude substances, I-tfe, II-tfe and III-tfe yielded single spots in thin-layer chromatography (detection under ultraviolet (UV) light at 254 and 360 nm); this showed that the esterification process took place without giving rise to side products.

Analytical level conversion of I and II into I-tfe and II-tfe

Five samples, containing 0.5, 1.25, 2.5, 5.0 and 10.0 μg of I and 5.0 μg of II (prepared by evaporation of methanolic solutions of I and II), were added to 0.2 ml of BF_3–2,2,2-trifluoroethanol. The test tubes were stoppered and heated at 60° for 15 min. After this time, excess reagent was evaporated off at 60° in nitrogen under reduced pressure, the tubes were rinsed with 0.2 ml of acetone and again dried to ensure collection of the sample in the lower portion of the tube; the operation was repeated by adding 0.1 ml acetone. When the solvent had evaporated, the residue was left under vacuum for an additional 5 min; 0.1 ml of a solution containing 200 μg of III-tfe (quantitation standard) per ml ethyl acetate were then added to the residue and a suitable aliquot of this solution submitted to gas–liquid chromatography (GLC) under the experimental conditions described below. The conversion percentages of I and II into I-tfe and II-tfe were calculated by comparing the GLC responses obtained by this procedure with those obtained by GLC of solutions at known concentrations of I-tfe, II-tfe and III-tfe. The results obtained by the procedure described are reported in Table II. They show that the average I/II conversion ratios fall within a narrow range (0.993±0.0092); II is therefore a suitable internal standard to control the conversion of I into I-tfe. The variability in the extent of conversion of I and II into their 2,2,2-trifluoroethyl esters is due to losses by volatilization occurring during the evaporation following derivatisation; in fact, when the evaporation time was prolonged, the yields of these esters dropped further, even if the ratio between the yields fell within the range mentioned above.

TABLE II

CONVERSION YIELDS OF I AND II INTO I-tfe AND II-tfe

Amount of compound in each sample (μg)		*Average* percentage recovery ± standard deviation*		*Average* recovery ratios ± standard deviation*
I	*II*	*I*	*II*	*I/II*
0.5	5.0	83.94±4.11	84.66±3.23	0.991±0.016
1.25	5.0	84.50±3.94	85.50±4.39	0.988±0.006
2.5	5.0	87.98±2.16	88.80±2.67	0.991±0.005
5.0	5.0	82.00±1.85	82.04±1.45	0.999±0.008
10.0	5.0	80.66±4.57	81.02±4.62	0.996±0.007
		Overall average ± standard deviation		0.993±0.009

* Mean of five determinations.

Extraction of I and II from plasma and urine and determination of their recoveries

Compounds I and II (internal standard) are quantitatively extractable with 4 volumes of diethyl ether from an aqueous buffer at pH <2 and with 0.1 volume 0.1 *N* NaOH from diethyl ether. They are also stable in 0.1 *N* HCl and 0.1 *N* NaOH. On the basis of these properties the following method was developed for their extraction from plasma and urine.

Volumes of 0.2, 0.5, 1.0, 2.0 and 4.0 ml of a methanolic solution of I and 2.0

ml of a methanolic solution of II (both at a concentration of 200 μg/ml) were transferred into 10-ml calibrated flasks and taken to volume with methanol. Volumes of 0.25 ml of these solutions were taken to dryness at 40° under a stream of nitrogen and 2 ml human plasma were added; each sample thus contained 0.5, 1.25, 2.5, 5.0 and 10.0 μg of I and 5.0 μg of II per ml plasma. In the same way, using methanolic solutions of suitable concentrations, urine samples containing 5.0, 10.0, 25.0 or 50.0 μg of I and 25.0 μg of II per ml were prepared. The recoveries of I were determined in the above plasma and urine concentration range on the basis of plasma and urinary levels in man after oral administration of 100 mg of I. After equilibration at 37°, plasma and urine samples were acidified by addition of 4 ml 0.1 *N* HCl and extracted by mechanical shaking for 15 min with 40 ml diethyl ether. The organic phase was washed with 5 ml distilled water and re-extracted with 5 ml 0.1 *N* NaOH; after washing with 40 ml diethyl ether and acidification with 1 ml 1 *N* HCl, I and II were extracted from the aqueous phase by mechanical shaking for 15 min with 40 ml diethyl ether. After washing with 5 ml distilled water, the ether was taken to dryness at 30° under a stream of nitrogen. To the residue was added 0.2 ml BF_3–2,2,2-trifluoroethanol and esterification was carried out as described earlier. The residue from the esterification was redissolved in 0.1 ml of a solution containing 200 μg of III-tfe per ml ethyl acetate. Of the solution thus obtained 1 μl was submitted to GLC. When plasma samples contained less than 1 μg/ml of I, the equivalent 2% of an extract of 2 ml plasma had to be submitted to GLC and the highest instrument sensitivity used. In these cases, a further purification step was added before the esterification. This consisted of partitioning the extraction residue between 1 ml methanol containing two drops of distilled water and 3 ml hexane. The lower, methanol phase was then taken to dryness and the residue esterified. As the internal standard (compound II) was added to the initial plasma or urine samples, the total recovery of extracts during the whole procedure was not necessary. Percentage recoveries of increasing quantities (0.5–10.0 μg) of I added to 1 ml human plasma are reported in Table III. Table IV gives the percentage recoveries of increasing amounts (5.0–50.0 μg) of the same compound added to 1 ml urine. These recoveries were determined by comparison of the GLC responses obtained by this procedure

TABLE III

RECOVERIES OF I AND II FROM PLASMA

Amount of compound added to each ml plasma sample (μg)		*Average* percentage recovery ± standard deviation*		*Average* recovery ratios ± standard deviation*
I	*II*	*I*	*II*	*I/II*
0.5	5.0	70.90 ± 2.27	72.13 ± 3.32	0.983 ± 0.013
1.25	5.0	77.43 ± 5.04	77.43 ± 4.34	1.000 ± 0.011
2.5	5.0	79.97 ± 3.25	81.23 ± 2.56	0.984 ± 0.014
5.0	5.0	79.88 ± 1.57	80.65 ± 2.37	0.991 ± 0.016
10.0	5.0	81.23 ± 2.19	81.67 ± 3.12	0.995 ± 0.016
		Overall average ± standard deviation		0.991 ± 0.015

* Mean of six determinations.

TABLE IV

RECOVERIES OF I AND II FROM URINE

Amount of compound added to each ml urine sample (μg)		*Average* percentage recovery ± standard deviation*		*Average* recovery ratios ± standard deviation*
I	*II*	*I*	*II*	*I/II*
5	25	73.20 ± 2.63	73.93 ± 2.58	0.990 ± 0.004
10	25	72.80 ± 1.52	73.70 ± 1.39	0.988 ± 0.004
25	25	71.95 ± 0.94	72.85 ± 0.95	0.988 ± 0.006
50	25	73.63 ± 2.52	75.02 ± 2.39	0.981 ± 0.007
		Overall average ± standard deviation		0.987 ± 0.006

* Mean of six determinations.

with those given by the GLC of solutions at known concentrations of I-tfe, II-tfe and III-tfe. On the basis of the results obtained, the following method was developed for determining I in human plasma and urine.

Determination of I in human plasma and urine

A 10.0-μg amount of compound II (internal standard) was added to 2 ml plasma or 1 ml urine containing unknown quantities of I. The sample was acidified with 4 ml 0.1 *N* HCl and processed as shown schematically in Fig. 1, which summarizes the extraction and derivative formation procedure reported in the preceding paragraph. In order to determine the total (free and glucuronide) amount of I present in urine, the samples were brought to pH 5.2 (acetate buffer) and incubated for 20 h at 37° with β-glucuronidase. After addition of internal standard and acidification,

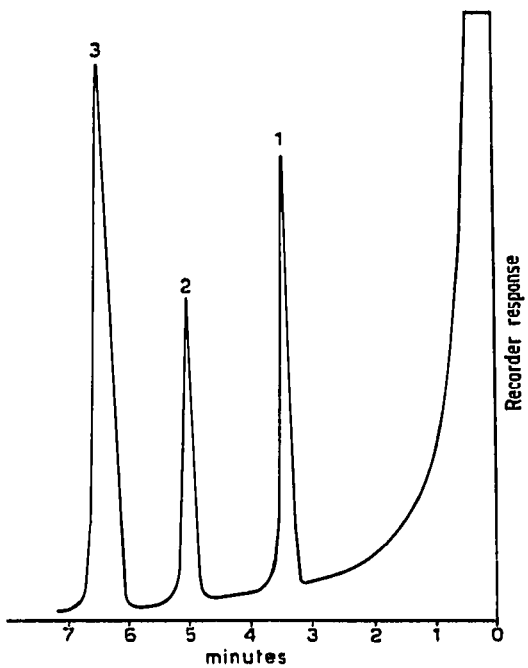
plasma (2 ml) or urine (1 ml) with added II (10 μg) and 0.1 *N* HCl (4 ml)
↓
extracted with diethyl ether (40 ml)
↓
organic phase: washed with water (5 ml) and extracted with 0.1 *N* NaOH (5 ml)
↓
NaOH phase: washed with diethyl ether (40 ml), acidified with 1 *N* HCl and extracted with diethyl ether (40 ml)
↓
organic phase: washed with water (5 ml) and dried
↓
residue: partitioned between methanol (1 ml) and hexane (3 ml)
↓
methanol (lower phase): dried
↓
residue: reacted (15 min at 60°) with BF_3–2,2,2-trifluoroethanol (0.2 ml)
↓
solution: dried
↓
residue: 0.1 ml quantitation standard solution (III-tfe) added and sample submitted to GLC

Fig. 1. Scheme for the determination of I in human plasma and urine.

urine was submitted to extraction and derivative formation procedure as described previously. This further step caused no interference in the subsequent GLC determination. Plasma and urine samples were frozen (−20°) and analysed for compound I up to three weeks after collection. Under these storage conditions no significant changes took place in plasma or urine values. The quantities of I in plasma and urine samples were deduced (as stated above) from the recovery of the internal standard added to the initial plasma and urine sample. Therefore the quantitation standard (III-tfe) added at the end of the extraction and derivative formation is used only to check the efficiency of these two steps.

Gas–liquid chromatography

The instrument employed in this investigation was a Carlo Erba Model GI equipped with flame ionization detector with a hydrogen flow-rate of 35 ml/min and an air flow-rate of 400 ml/min. A coiled glass column (2 m × 4 mm I.D.) packed with 1% SE-54 on silanized Chromosorb W AW, 60–80 mesh, was used. Coating of the support was effected by the filtration technique. Nitrogen was used as carrier gas at a flow-rate of 60 ml/min. The temperatures were as follows: column 240° and injector port and detector 260°. Fig. 2 shows a gas chromatogram of the reference compounds employed in this research. Fig. 3 shows the calibration curve for I-tfe



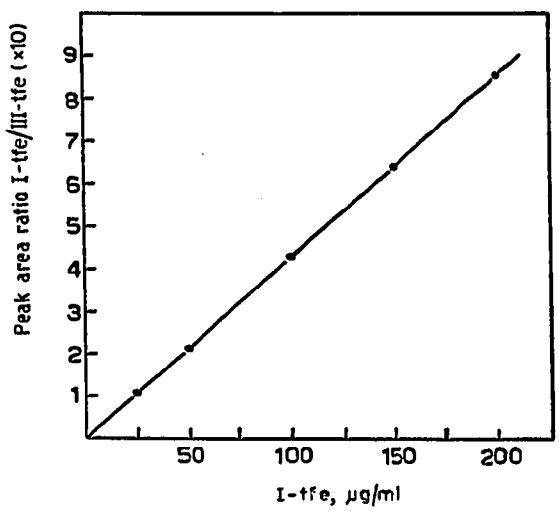


Fig. 2. Gas chromatogram of standard I-tfe (1), II-tfe (2) and III-tfe (3) on a 2-m glass column packed with 1% SE-54 on silanized Chromosorb W AW 60–80 mesh. Column temperature, 240°; carrier gas (nitrogen) flow-rate, 60 ml/min; chart speed, 2 cm/min.

Fig. 3. Linearity of GLC response (peak area I-tfe/peak area III-tfe) *versus* concentration of I-tfe.

obtained by GLC of ethyl acetate solutions containing increasing amounts of I-tfe and a fixed amount (200 μg/ml) of III-tfe (quantitation standard). A linear relationship passing through the origin is obtained between the ratio of peak area I-tfe to peak area III-tfe and the concentration of I-tfe.

The area of the chromatographic peaks was calculated by multiplying the peak height by the width of the peak at half height.

Specificity and sensitivity of the method

Fig. 4 shows the gas chromatogram relating to the determination of I in the plasma extract of a subject treated with 100 mg of I. Fig. 5 shows the gas chromatogram of a blank plasma extract of the same subject. In the chromatogram of the blank plasma there are no peaks interfering with those of the substance under examination and the internal and quantitation standards. No such interference occurs when the equivalent of 2% of an extract of 2 ml plasma or of 1% of an extract of 1 ml urine are subjected to GLC. Under these conditions I can be determined in plasma and urine at levels as low as 0.2 and 2.5 μg/ml, respectively.

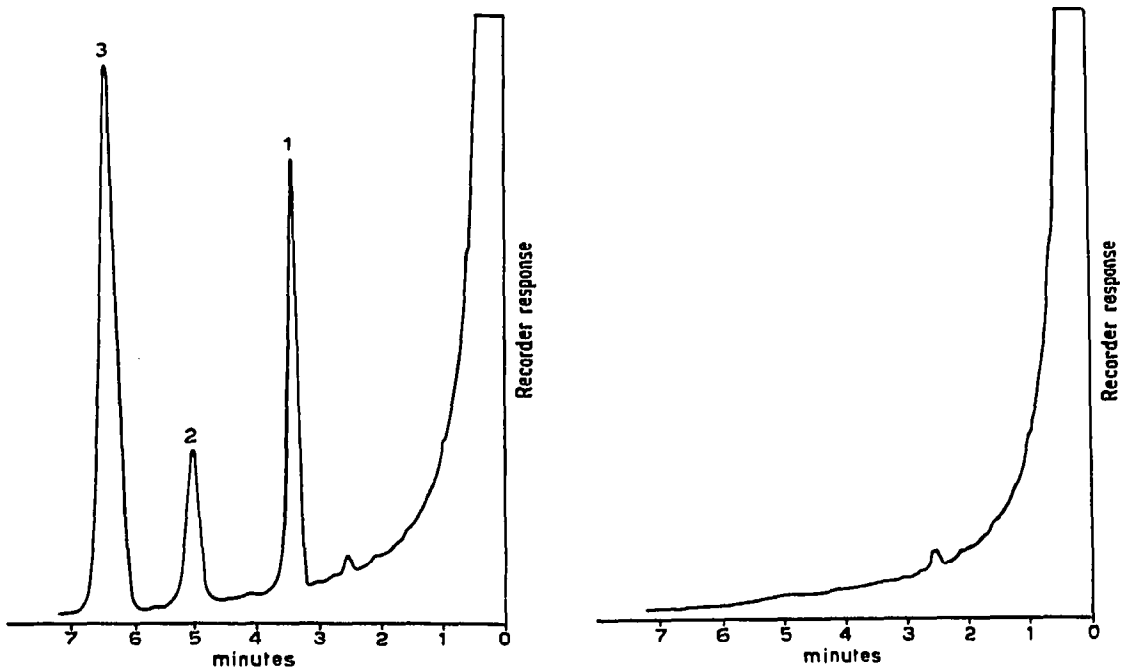


Fig. 4. Gas chromatogram of a plasma extract of a human subject treated with 100 mg of compound I. GLC conditions as in Fig. 2. 1 = I-tfe; 2 = II-tfe; 3 = III-tfe.

Fig. 5. Gas chromatogram of an extract of 2 ml blank human plasma. GLC conditions as in Fig. 2.

Precision and accuracy of the method

The data in Table III show that a linear relationship (correlation coefficient, r=0.999 for a probability, $<$0.01) exists between the amount of I recovered from plasma, calculated with reference to the recovery of II, and the amount added. The

regression coefficient (±standard deviation) of this relationship is 0.995±0.0042 and the intercept (± standard deviation) is −0.011±0.022. Table IV shows that also in the case of urine the amount of I recoverd and calculated on the basis of the recovery of II is related to the amount added by a linear relationship (r=0.999 for p<0.01), the regression coefficient of which is 0.980±0.0022 and the intercept 0.087±0.064.

RESULTS

Fig. 6 shows the plasma level/time curve of I following oral administration of 100 mg to a subject. The amounts of free compound I excreted by the same subject in urine collected 0–4, 4–8, 8–12 and 12–24 h following the above treatment were 6.40, 1.88, 0.58 and 1.10 mg, respectively. Those of total (free and glucuronide) compound I excreted in the same time intervals were 50.60, 14.95, 6.70 and 8.05 mg, respectively. Work[3] on the pharmacokinetics of compound I in man carried out with this GLC method and radioisotopic techniques has shown that this substance is rapidly and completely absorbed from the gastrointestinal tract after oral administration in capsules (peak plasma levels between 30 min and 2 h) and that its $t_{\frac{1}{2}}\beta$ ranges between 2.16 and 5.40 h.

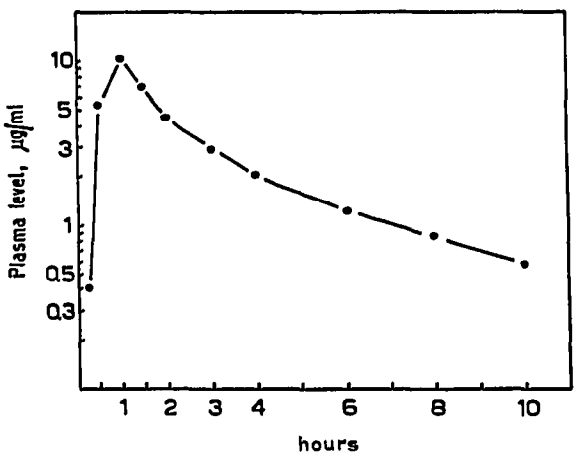


Fig. 6. Plasma levels (µg/ml) of compound I in a subject after oral administration of 100 mg.

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