Journal of Chromatography, 310 (1984) 431—437
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2201

Note

Determination of aminoglutethimide and N-acetylaminoglutethimide in human plasma by high-performance liquid chromatography

G. MENGE* and J.P. DUBOIS

Ciba-Geigy Limited, Pharma Research and Development, Basle (Switzerland)

(First received January 31st, 1984; revised manuscript received May 10th, 1984)

Aminoglutethimide (AG) was shown to inhibit steroidgenesis, and has therefore found use as an alternative in the therapy of breast carcinoma [1].

Since the acetylation of AG is acetylator phenotype dependent [2] and N-acetylaminoglutethimide (N-AcAG) is the major metabolite excreted in urine, we developed a high-performance liquid chromatographic (HPLC) assay for the simultaneous measurement of AG and N-AcAG in human plasma using 2-(*p*-N-acetylaminophenyl)-2-methylglutarimide as internal standard (Fig. 1).

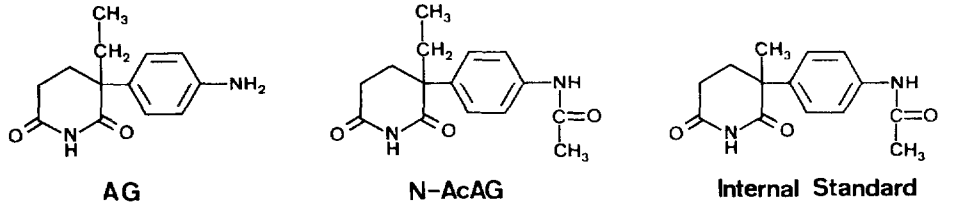

Fig. 1. Structures of AG [aminoglutethimide, 2-(*p*-aminophenyl)-2-ethylglutarimide], N-AcAG [N-acetylaminoglutethimide, 2-(*p*-N-acetylaminophenyl)-2-ethylglutarimide], and internal standard [2-(*p*-N-acetylaminophenyl)-2-methylglutarimide].

The HPLC methods available for the determination of AG in plasma [3] or for AG and metabolites in urine [2] worked without an internal standard. Only recently did we learn of a method [4] offering the possibilities we were looking for during the development of our method.

EXPERIMENTAL

Chemicals

All solvents and reagents were of analytical grade (Fluka, Buchs, Switzer-

0378-4347/84/$03.00

land, and E. Merck, Darmstadt, F.R.G.) and were used without further purification. ^{14}C-Labelled AG and N-AcAG, as well as the internal standard 2-(*p*-N-acetylaminophenyl)-2-methylglutarimide, originated from Ciba-Geigy, Basle, Switzerland (Fig. 1). Water was deionized, distilled in a glass apparatus and filtered through a 4.5-μm Millipore® (Waters Assoc., Milford, MA, U.S.A.) filter before use.

Apparatus

The chromatographic system consisted of a Vista 5040 ternary liquid chromatograph and the Vista 401 data system (both from Varian, Palo Alto, CA, U.S.A.). The WISP (Waters Assoc.) was used for automatic sample injection and a variable-wavelength detector Spectroflow SF 773 (Kratos, Ramsey, NJ, U.S.A.) served to monitor the eluent at 235 nm. The column (100 mm × 4.8 mm I.D.) was packed with Nucleosil C_{18}, 5 μm (Macherey-Nagel, Düren, F.R.G.). The mobile phase (acetonitrile—methanol—water, 5:20:75, v/v/v) was used at a flow-rate of 1 ml/min and a column temperature of 30°C.

Preparation of standard solutions

Stock solutions of AG and N-AcAG were prepared by dissolving 10 mg of each compound in 100 g of water containing 10% ethanol. Aliquots of these stock solutions were combined and diluted with water to yield a working solution containing 1 μg/g of each of the two compounds. This solution served to prepare spiked plasma samples for calibration curves and recovery analysis.

A stock solution of internal standard was prepared by dissolving 10 mg of 2-(*p*-N-acetylaminophenyl)-2-methylglutarimide in 100 ml of water containing 10% ethanol. An aliquot was diluted with water to yield a working solution containing 1 μg/ml. To each analytical plasma sample 0.5 μg of the internal standard was added using a Repipette® (Labindustries, Berkeley, CA, U.S.A.) sampler.

Both working solutions, if kept at 5°C, were found to be stable for at least four weeks.

Procedure

Weigh 0.5 g of plasma (AC 100 balance, Mettler, Greifensee, Switzerland) into a ground-glass stoppered centrifuge tube and dilute with 1 g of water. Weighing of plasma aliquots was preferred because of higher precision and better documentation. (For calibration curves and recovery analysis add 1 g of aqueous solution containing known amounts of the AG and N-AcAG working solution instead of 1 g of water.) Add 0.5 ml (Repipette sampler) of the internal standard working solution and shake for 5 min (DSG 304 vertical mixer; Heidolph, Kelheim, F.R.G.) to mix homogeneously the internal standard and analytical sample. Then add 7 ml of extraction solvent (diethyl ether—dichloromethane, 2:1, v/v). Seal the extraction tube with a stopper and shake for 12 min (HT horizontal shaker at 200 rpm; Infors, Basle, Switzerland). Centrifuge for 5 min (Multex centrifuge at 940 *g*; MSE, Crawley, U.K.). For easy separation freeze the aqueous phase by dipping the lower part of the tube into a dry ice—ethanol mixture. Transfer the organic layer into a 16 × 40 mm disposable glass ampoule and evaporate the organic solvents by

gently blowing nitrogen into the ampoule at 40° C. Reconstitute the residue in 0.2 ml of the mobile phase and transfer the solution into a micro injection vial.

A ternary solvent system was used since adding 5% acetonitrile improved the resolution by selectively lowering the retention of the biological background. Retention times of AG and N-AcAG were 8.9 and 12.1 min, respectively. The retention time of the internal standard was 6.1 min. Typical chromatograms are shown in Fig. 2.

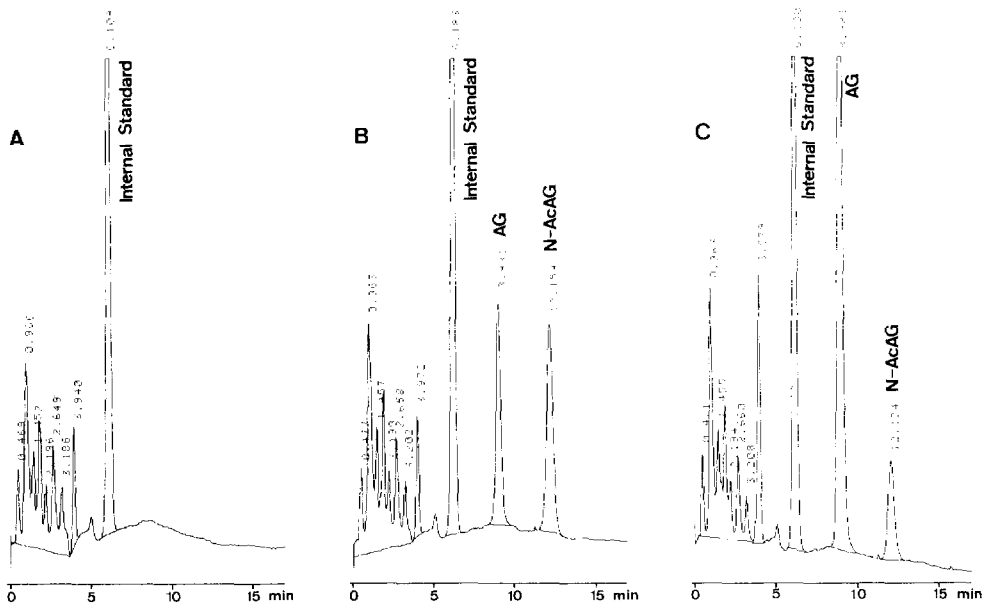

Fig. 2. (A) Chromatogram obtained from analysis of a blank plasma sample containing internal standard. (B) Chromatogram obtained from analysis of a spiked plasma sample containing 0.4 μg/g each of aminoglutethimide (AG) and N-acetylaminoglutethimide (N-AcAG). (C) Chromatogram obtained from analysis of a plasma sample obtained from a healthy volunteer 1 h after administration of a 250-mg tablet of aminoglutethimide.

Calibration

To establish calibration curves, plasma samples with known concentrations were prepared by adding working solution of AG and N-AcAG to 0.5 g of blank human plasma. After addition of 0.5 μg of the internal standard, the samples were processed as described above.

A 50-μl volume of the reconstituted extract of each sample was injected into the chromatographic system. The peak area values of the compounds were divided by the peak area value of the internal standard and the resulting ratios (F_X) plotted against initial concentrations given. By regression analysis the following terms for calibration curves in the range 0—2.0 μg/g were obtained: $F_X = 1.004 \cdot X$, $s_Y = 0.023$, $r = 0.9996$ for AG; and $F_X = 1.073 \cdot X$; $s_Y = 0.006$, $r = 0.9999$ for N-AcAG. X denotes the independent variable, i.e. the concentrations of AG and N-AcAG in plasma in units of μg/g. F_X denotes the dependent variable, i.e. the ratio of the peak area values; s_Y and r denote the estimated standard deviation and the coefficient of correlation [5].

In routine analyses of large series of plasma samples single-point calibration was used to calculate the concentrations of AG and N-AcAG.

RESULTS

Extractability

To isolate the compounds to be analysed from plasma, solvent extraction after dilution of the plasma samples was found to be suitable. Besides the high purification power the single-step solvent extraction offers the possibility of concentrating the extract prior to injection.

The partition of 1 μg of ^{14}C-labelled aminoglutethimide between buffered plasma samples (0.5 ml of plasma and 1.5 ml of buffer) and organic extraction phase (7 ml of diethyl ether—dichloromethane, 2:1, v/v) was measured radiometrically in dependence on the pH (Fig. 3). In the pH range 4—10 the amount of AG in the organic phase was not dependent on the pH (pK_{a1} of AG = 4.2, pK_{a2} of AG = 11.9).

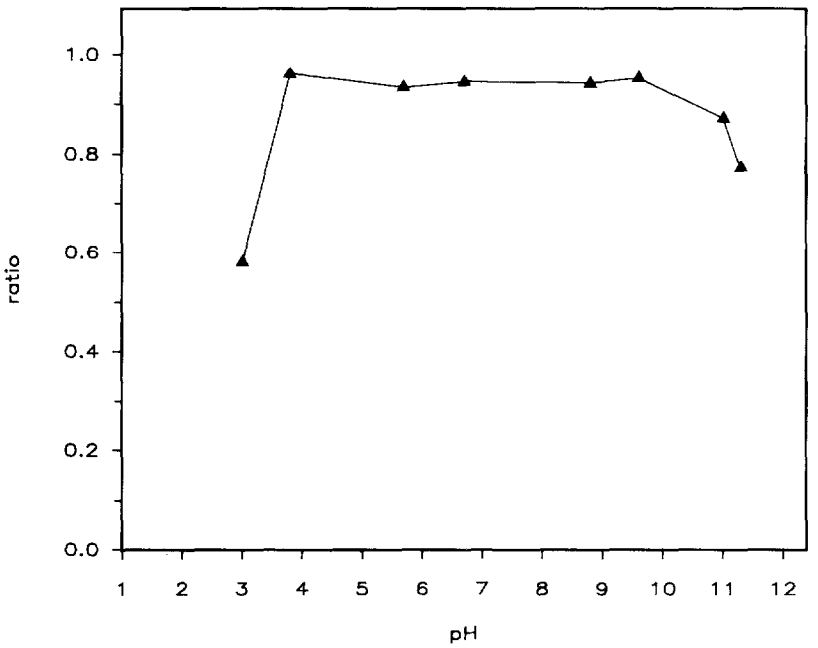

Fig. 3. Partition of 1 μg of ^{14}C-labelled aminoglutethimide between buffered plasma samples (0.5 ml plasma and 1.5 ml buffer) and organic extraction phase (7 ml of diethyl ether—dichloromethane, 2:1, v/v) in dependence on the pH. Aminoglutethimide: pK_{a1} = 4.2, pK_{a2} = 11.9.

Since the extractable biological background could not be drastically decreased at any pH, the plasma samples were extracted at their physiological pH.

The extent of the extractabilities of AG, N-AcAG and the internal standard from plasma at physiological pH was determined by comparison of the peak area after direct injection of known amounts with the peak area resulting after injection of the reconstituted extracts of spiked plasma samples that underwent

the whole work-up procedure. The extractabilities (mean ± S.D., n = 20) measured at ten different concentration levels covering the range 0.004—2.0 μg/g were 71 ± 5.4% for AG, 91 ± 3.3% for N-AcAG and 75 ± 6.7% for the internal standard.

Precision and accuracy

Recovery of spiked plasma samples was always analysed together with series of analytical samples. Four samples each containing 0.4 and 0.8 μg/g of both AG and N-AcAG were analysed in four independent analytical series. The results of these recovery analyses are given in Table I.

The standard deviations expressed in per cent of the total mean were 8.7% and 5.6% for AG and N-AcAG, respectively, at the concentration level 0.4 μg/g. The respective values were 5.3% and 3.1% at the 0.8 μg/g concentration level.

The mean deviations from given concentrations were between —8.7% and +10.1% for AG and between —7.2% and +1.9% for N-AcAG when a concentration of 0.4 μg/g was given. At the concentration level of 0.8 μg/g the mean

TABLE I

RESULTS OF RECOVERY ANALYSES USING SPIKED PLASMA SAMPLES

The analyses were performed on four different days with a single-point calibration procedure.

	AG found (ng/g)				N-AcAG found (ng/g)			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
	Given 400 ng/g							
	403.4	373.0	453.4	393.2	390.6	402.0	401.8	402.0
	381.6	344.4	438.0	376.2	393.8	365.4	390.8	402.6
	349.6	342.8	440.4	386.4	349.4	377.4	407.2	388.8
	373.8	400.4	429.4	385.8	351.4	378.2	430.8	414.2
Mean	377.1	365.2	440.3	385.4	371.3	380.8	407.7	401.9
± R.S.D.*	± 5.9	± 7.5	± 2.3	± 1.8	± 6.5	± 4.0	± 4.1	± 2.6
Accuracy**	—5.7%	—8.7%	+10.1%	—3.7%	—7.2%	—4.8%	+1.9%	+0.5%
	Given 800 ng/g							
	843.6	794.9	893.8	808.8	750.8	763.8	809.6	776.4
	836.4	869.2	879.8	802.6	762.8	784.4	815.4	779.2
	848.8	798.4	861.2	878.8	756.4	750.0	806.6	824.4
	825.8	717.2	860.0	819.8	766.2	759.6	800.0	793.6
Mean	838.7	794.9	873.7	827.5	759.1	764.5	807.9	793.4
± R.S.D.	± 1.2	± 7.8	± 1.9	± 4.2	± 0.9	± 1.9	± 0.8	± 2.8
Accuracy	+4.8%	—0.6%	+9.2%	+3.4%	—5.1%	—4.4%	+1.0%	—0.8%

*R.S.D. = $\frac{\text{S.D.}}{\text{Mean}} \times 100\%$.

**Accuracy = $\frac{\text{Found} - \text{given}}{\text{Given}} \times 100\%$.

deviations were between —0.6% and +9.2% for AG and —5.1% and +1.0% for N-AcAG.

The limit of quantitation was at least 0.2 μg/g. At this concentration level the relative standard deviations (R.S.D.) were 6.2% for AG and 4.6% for N-AcAG after analysis of four spiked plasma samples. The accuracy was +1.2% for AG and —1.1% for N-AcAG.

The detection limit was found at a concentration level of 0.02 μg/g. With parameters adjusted for the range 0—2.0 μg/g the integrator failed to integrate the area of the still visible peak at 0.02 μg/g.

Plasma concentration of aminoglutethimide and of its N-acetyl metabolite

After administration of one 250-mg tablet of aminoglutethimide to a healthy volunteer, the plasma concentrations of unchanged AG reached a maximum level of 2.4 μg/g 2 h after intake of the dose and dropped to 0.5 μg/g within 24 h (Fig. 4).

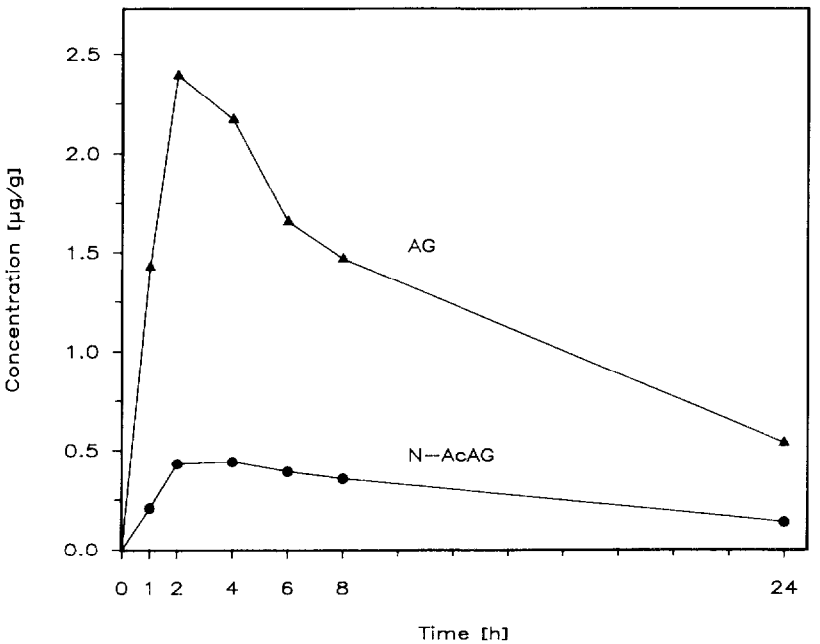

Fig. 4. Concentrations of aminoglutethimide (AG) and of N-acetylaminoglutethimide (N-AcAG) in plasma after administration of 250 mg of aminoglutethimide to a healthy volunteer, typed as slow acetylator using sulfadimidine.

The elimination half-life of aminoglutethimide was 10.4 h. This value was calculated by semilogarithmic—linear regression analysis using the concentrations of AG measured between 4 and 24 h after administration of the dose.

The N-acetyl metabolite showed a plateau concentration of about 0.4 μg/g between 2 and 4 h after administration; 24 h after ingestion of the dose 0.14 μg/g was measured.

Since the therapeutic dose is 1 g of AG per day, higher plasma levels of AG and of N-AcAG will have to be measured in patients.

CONCLUSION

The HPLC assay described for simultaneous analysis of aminoglutethimide (AG) and N-acetylaminoglutethimide (N-AcAG) using 2-(*p*-N-acetylaminophenyl)-2-methylglutarimide as internal standard permits the quantitation of plasma levels as they emerge after a single oral dose of 250 mg of aminoglutethimide.

ACKNOWLEDGEMENT

We thank Mr. G. Musslin for excellent technical assistance.

REFERENCES

1 R.W. Elsdon-Dew, I.M. Jackson and G.F.B. Birdwood (Editors), Aminoglutethimide — An Alternative Endocrine Therapy for Breast Carcinoma (International Congress & Symposium Series, No. 53), The Royal Society of Medicine, London, 1982.
2 R.C. Coombes, A.B. Foster, S.J. Harland, M. Jarman and E.C. Nice, Brit. J. Cancer, 46 (1982) 340.
3 B.A. Robinson and F.N. Cornell, Clin. Chem., 29 (1983) 1104.
4 C.O. Ruud, J.S. Kovach, M.E. Sanders and M.M. Ames, Proc. Amer. Ass. Cancer Res., 24 (1983) 288.
5 R.B. Davis, J.E. Thompson and H.L. Pardue, Clin. Chem., 24 (1978) 611.