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Determination of Aminoglutethimide and *N*-Acetylaminoglutethimide in Human Plasma by Reversed-Phase Liquid Chromatography

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Summary: A liquid chromatographic method for the determination of aminoglutethimide and *N*-acetylaminoglutethimide in human plasma is described. The assay involved precipitation of the plasma proteins using a mixture of acetonitrile and perchloric acid, without an extraction procedure. The supernatant was subjected to chromatography on a 3-μm ODS Hypersil column eluted isocratically with 11% acetonitrile in 100 m*M* ammonium formate buffer, pH 3.5. The absorbance was routinely recorded at 242 nm. The standard curves were linear in the range of 0.1–100 μg/ml, and the lower detection limit was ~0.1 μg/ml for aminoglutethimide and its plasma metabolite *N*-acetylaminoglutethimide. The precision of the method, given as the coefficient of variation, was 3.9%. With this method, it was determined that aminoglutethimide and *N*-acetylaminoglutethimide were present in the plasma of patients receiving single-dose or continuous treatment with aminoglutethimide for breast cancer. No *N*-formylaminoglutethimide or nitroglutethimide could be demonstrated in the plasma from these patients. Interference from several drugs commonly given to patients with breast cancer was ruled out. **Key Words:** Aminoglutethimide—*N*-Acetylaminoglutethimide—High-performance liquid chromatography.

Aminoglutethimide (AG), a derivative of the sedative drug glutethimide, has been found useful in the palliative treatment of breast cancer (1). The therapeutic activity of this agent has been attributed partly to its inhibitory effect on steroid aromatase, a key enzyme involved in the biosynthesis of estrogens (2).

AG is among those drugs that are polymorphically acetylated in humans (3). Thus, its effect, as well as some side effects (1), may be related to the acetylator phenotype of a particular patient treated with this drug.

The investigation of the possible relationship between the metabolic fate of AG and the adverse effects of this drug necessitated the development of a simple and sensitive method for the determination of AG and its metabolites in human plasma.

Colorimetric determination has been the most widely used method for measurement of AG in body fluids (4), but lack of specificity and high blank values are major disadvantages of this assay. Recently, AG and *N*-acetylaminoglutethimide (*N*-acetyl-AG) were determined in human plasma by high-performance liquid chromatography (HPLC) (5), but the methods have not been characterized in detail.

The present paper describes a procedure for the determination of AG and its plasma metabolite *N*-acetyl-AG in human plasma. The method is based on extraction with a mixture of acetonitrile and perchloric acid, followed by reversed-phase liquid chromatography on 3-μm ODS columns.

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MATERIALS AND METHODS

Chemicals and Drugs

HPLC-grade acetonitrile was purchased from Rathburn Chemicals, Ltd. (Peeblesshire, Scotland) analytical-grade perchloric acid was purchased from Merck (Darmstadt, F.R.G.). AG, *N*-acetyl-AG, and nitroglutethimide were gifts from Ciba-Geigy (Basel, Switzerland). *N*-Formyl-AG was prepared from AG by formylation in formic acid, followed by crystallization (3). The compound was homogeneous, as judged by reversed-phase liquid chromatography in various systems. ODS Hypersil 3-μm microparticle medium for reversed-phase chromatography was from Shandon Southern Products Ltd. (Cheshire, U.K.) and Pelliguard LC-18 (40 μm) from Supelco, Inc. (Bellefonte, PA, U.S.A.). The analytical column was slurry-packed with Hypersil at 9,000 psi, using a Shandon column packer. The guard column was dry-packed and subjected to mechanical compression, as described previously (6).

Standards

AG, *N*-acetyl-AG, *N*-formyl-AG, and nitroglutethimide were dissolved in 50% CH_3CN and sonicated for 10 min. The compounds were diluted to known concentrations in the same solution.

Sample Processing

AG, *N*-acetyl-AG, *N*-formyl-AG, and nitroglutethimide were added to human serum at concentrations from 0.1 to 100 μg/ml. Three procedures were evaluated for the extraction of AG and its metabolites from human plasma:

(a) Perchloric acid was added to the plasma (final concentration 0.4 N), and the precipitated protein removed by centrifugation. The acid was then neutralized to pH 7.0 by addition of 1.2 N $KHCO_3$/1.44 N KOH. After 15 min at 0°C, the insoluble potassium perchlorate was removed by centrifugation.

(b) Plasma was mixed with an equal volume of acetonitrile, and the precipitated protein was removed by centrifugation. The supernatant was transferred to sample vials, which were capped, and the samples were analyzed immediately.

(c) Plasma was mixed with an equal volume of a solution containing acetonitrile (50%) and perchloric acid (0.8 N). The supernatant was transferred to sample vials, which were capped, and the samples were analyzed immediately.

Instruments

A Spectra-Physics SP 8700 solvent delivery system was connected to a Perkin-Elmer ISS 100 autosampler for HPLC. The absorbance was routinely recorded at 242 nm, using a variable wavelength detector from Kratos, model Spectroflow 773. For the optimization of the method and identification of the chromatographic peaks, a photodiode array detector (Hewlett Packard model 1040 A) was used. The chromatographic profiles were recorded and integrated by a reporting integrator (Spectra Physics model SP 4270).

HPLC

Samples of 25 μl were injected into a 10-cm 3-μm ODS Hypersil column equipped with a guard column (2.5 cm). The temperature of the sample tray was 23°C, and the amount of AG and *N*-acetyl-AG in the samples remained stable at this temperature for at least 12 h. The column was routinely eluted isocratically with 11% acetonitrile in 100 m*M* ammonium formate buffer, pH 3.5, for the determination of AG, *N*-formyl-AG, and *N*-acetyl-AG. Other mobile phases used include 8–20% acetonitril in 15 m*M* acetate buffer, pH 4.5, or 10 m*M* phosphate buffer, pH 6.0. Elution of nitroglutethimide required a stepwise increase in the acetonitrile concentration from 11 to 23% after 16 min. The flow rate was routinely 2 ml/min for unattended analysis. The retention times were ~4.5 min (AG), 14 min (*N*-formyl-AG), 17 min (*N*-acetyl-AG), and 28 min (nitroglutethimide).

RESULTS

Evaluation of the Extraction Procedures

Almost complete recovery of AG, *N*-acetyl-AG, and nitroglutethimide added to plasma was obtained when serum proteins were precipitated with acetonitrile or perchloric acid in combination with acetonitrile. Deproteinization with perchloric acid resulted in low recovery of AG and its metabolites (Table 1). The combination of perchloric acid and acetonitrile gave reduced recovery of *N*-formyl-AG. However, this combination gave somewhat longer column life than did acetonitrile alone. In this solution, the decomposition of *N*-formyl-AG to AG

TABLE 1. *Recovery of exogenously added aminoglutethimide (AG) and metabolites from serum*

Extraction procedure	Recovery (%)		
	AG	*N*-Formyl-AG	*N*-Acetyl-AG
$HClO_4/CH_3CN$[a]	101.0 ± 3.2	41.4 ± 2.3	98.5 ± 1.5
CH_3CN[b]	102.6 ± 2.4	93.2 ± 1.3	100.7 ± 1.1
$HClO_4$[c]	99.2 ± 4.6	0.0	48.7 ± 2.8

[a] Extraction was carried out as described in Methods using a mixture containing 50% CH_3CN in 0.8 N $HClO_4$.
[b] Extractions were carried out using 100% CH_3CN.
[c] Extractions were carried out using 0.8 N $HClO_4$.
All values are mean ± SEM for five determinations, and are calculated relative to the recovery of the pure compounds dissolved in 50% acetonitrile.

was a first-order process with a rate constant of 0.52 h^{-1}, whereas *N*-acetyl-AG was stable.

Reversed-Phase Liquid Chromatography

When the ODS column was eluted with a mobile phase containing 100 m*M* ammonium formate (pH 3.5), neutralization of the sample containing perchloric acid was not required. The guard column was routinely subjected to mechanical compression (after each 100 injections) to prevent peak splitting during unattended analysis, as described in detail previously (6).

AG, *N*-formyl-AG, and *N*-acetyl-AG were separated from each other and from interfering material on a 3-μm ODS Hypersil column, eluted isocratically with 11% acetonitrile in 100 m*M* ammonium formate, pH 3.5. Inclusion of nitroglutethimide in the chromatogram required an increase in the acetonitrile concentration of the mobile phase. No interfering peaks were detected in serum from patients not receiving AG (Fig. 1). The absorbance was routinely recorded at 242 nm.

In serum from patients given a single dose of AG (250 mg) or daily doses of the drug (250 mg q.i.d.), AG and *N*-acetyl-AG could be demonstrated. *N*-formyl-AG and nitroglutethimide were not detected (Fig. 1). The assay for nitroglutethimide was based on monitoring the absorbance at 272 nm, corresponding to the absorption maximum of this compound (data not shown).

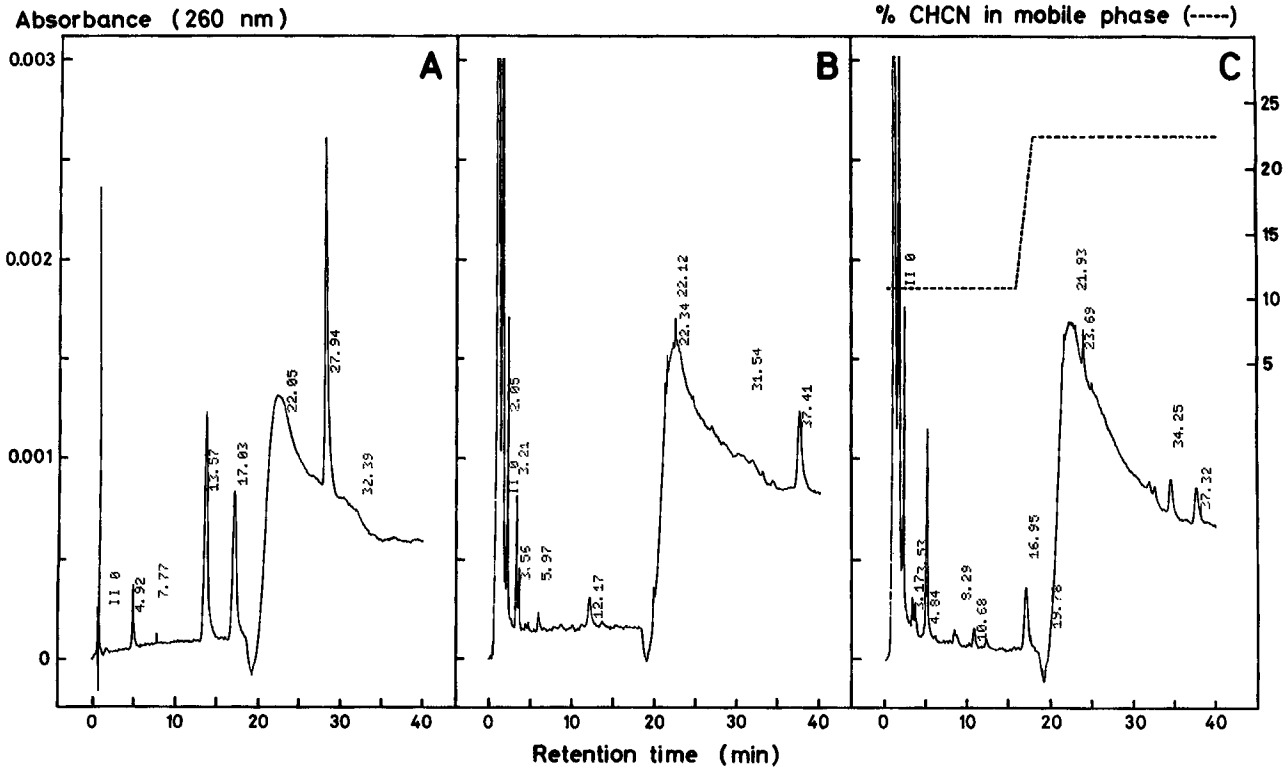

FIG. 1. High-performance liquid chromatographic analysis of AG and its metabolites in human serum. A: Elution profile of standards. AG (retention time of 4.92 min), *N*-formyl-AG (13.57 min), *N*-acetyl-AG (17.03 min), and nitroglutethimide (27.94 min). B: Profile obtained with plasma from a person not treated with AG or other drugs. C: Chromatogram obtained with serum from a patient treated with AG. The reversed-phase column was eluted isocratically with 11% acetonitrile in 100 m*M* ammonium formate buffer, pH 3.5, for the first 17 min. The concentration of acetonitrile in the mobile phase was then increased to 22%.

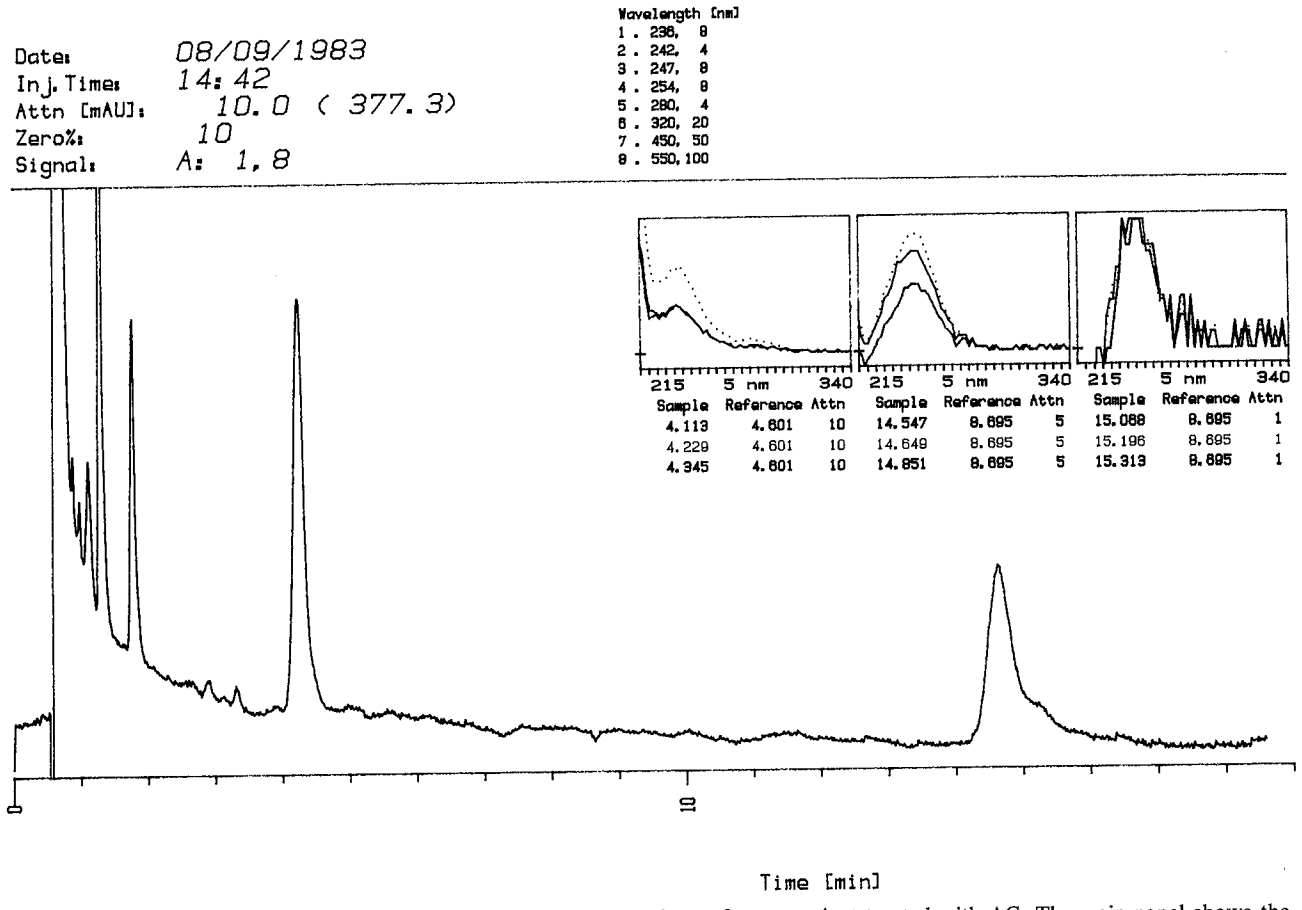

FIG. 2. Absorption spectra of the chromatographic peaks in the plasma from a patient treated with AG. The main panel shows the chromatogram (236 nm, 8-nm band width). The inset shows the absorption spectrum of the peaks tentatively identified as AG (left), *N*-acetyl-AG (middle), and the tailing of the *N*-acetyl-AG peak (right). The spectra were taken at the up-slope, apex, and down-slope of the peak, as indicated below the spectra. The column was eluted isocratically with the mobile phase described in the legend to Fig. 1.

Identification and Purity of the Chromatographic Peaks

AG and *N*-acetyl-AG were identified in plasma by their retention times in various systems (6–15% acetonitrile in 15 m*M* acetate buffer, pH 4.5, 100 m*M* ammonium formate buffer, pH 3.5, or 10 m*M* phosphate buffer, pH 6.0). In addition, absorption spectra of the AG and *N*-acetyl-AG peaks were taken on the up-slope, at the maximum, and on the down-slope of the peaks, using a photodiode array detector for HPLC (Fig. 2). The spectra of each separate peak was identical (Fig. 2) and the same as the spectra obtained with spectrophotometry (data not shown). AG and *N*-acetyl-AG showed absorption maxima at 236 and 247 nm, respectively (Fig. 2).

Interference with Various Drugs

Several drugs commonly given to patients with breast cancer were screened for interference by analysis of serum from patients treated with these drugs but not with AG. The drugs tested are listed in Table 2. In plasma from patients treated with naproxen, there was an ultraviolet (UV) absorbing peak, which cochromatographed with *N*-acetyl-AG in several isocratic and gradient systems at pH 6.0. No interference from this material was observed when the pH of the mobile phase was decreased to 4.5 or 3.5.

TABLE 2. *Drugs tested for interference with the aminoglutethimide assay*

Commercial name	Active substance
Apocillin	Phenoxymethylpenicillin
Apodorm	Nitrazepam
Daonil	Glibenclamide
Decadron	Dexamethasone
Digitrin	Digitoxin
Diural	Furosemide
Esidrex-K	Hydrochlorothiazid
Felden	Piroxicam
Inderal	Propranolol
Indocid	Indomethacin
Largactil	Chlorpromazine
Marevan	Sodium warfarin
Methadone	Methadone hydrochloride
Morfin	Morphine
Naprosyn	Naproxen
Nolvadex	Tamoxifen
Paralgin Forte	Paracetamol/codeine phosphate
Prednisone	Prednisone
Rohypnol	Flunitrazepam
Temgesic	Buprenorphine hydrochloride
Valium	Diazepam

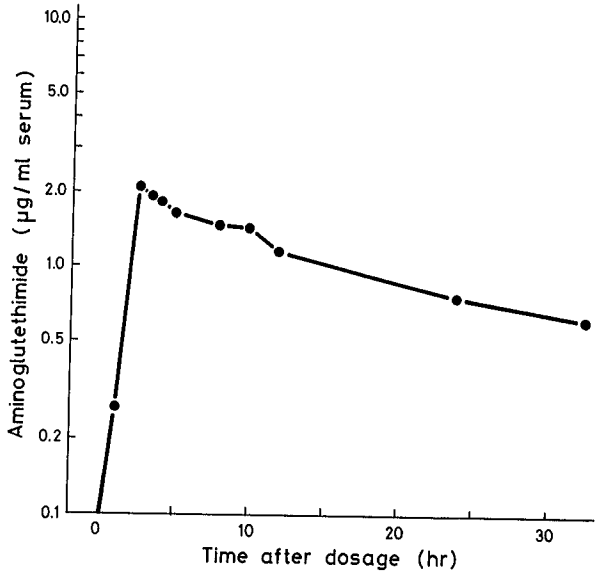

FIG. 3. Serum concentration curve for AG after oral administration (250 mg single dose).

Standard Curves, Sensitivity, and Precision of the Method

The standard curves for AG, *N*-formyl-AG, and *N*-acetyl-AG were linear in the range of 0.1–100 µg/ml, and the detection limit of the method was about 0.1 µg/ml for all three compounds. At a concentration of 0.5 µg/ml, the reproducibility, expressed as coefficient of variation, was 3.9 and 2.6%, for AG and *N*-acetyl-AG, respectively.

Plasma Concentration Curves

The present method was used in our laboratory for the determination of AG and its metabolite *N*-acetyl-AG in serum from patients receiving single-dose or continuous treatment with AG. A plasma concentration curve after a single dose is shown in Fig. 3. The serum half-life was about 7 h for AG.

DISCUSSION

This report describes an isocratic HPLC method for the determination of AG and *N*-acetyl-AG in human serum. *N*-Formyl-AG eluted between AG and *N*-acetyl-AG, but this metabolite could not be detected in serum from any patient receiving either single-dose or continuous AG treatment. Inclusion of nitroglutethimide required gradient elution and prolonged analysis time (Fig. 1), but the amount of this metabolite was below the detection limit. Therefore, the present method was optimized for the detection of AG and *N*-acetyl-AG in human serum, and the absorbance was recorded at 242 nm during routine analysis.

It should be noted that the retention time of *N*-acetyl-AG (Fig. 1), which corresponds to about 25 ml of mobile phase, is long, relative to the short column used. This result was obtained by reducing the acetonitrile concentration of the mobile phase to 11%, which was required to separate AG, *N*-acetyl-AG (and *N*-formyl-AG), and uv-absorbing material present in human serum (Fig. 1). In this system, there was no interference from several drugs commonly given to patients with breast cancer (Table 2).

The analysis time could be shortened by increasing the flow rate from 2 to 3 ml/min. However, the column was routinely eluted to 2 ml/min, to avoid build-up of overpressure during unattended analysis.

The present method involves precipitation of the plasma proteins using a mixture of perchloric acid and acetonitrile. No laborious extraction procedure was required. The mixture of acid and acetonitrile was preferred as an extraction medium because the samples obtained using perchloric acid or acetonitrile alone were more damaging to the column,

leading to shorter column life. This may be related to precipitation of material absorbing to the stationary phase in the presence of an extraction solution containing both perchloric acid and acetonitrile. Furthermore, the high buffer capacity of the mobile phase may counteract the effect of low pH on the bonded phase (7). The simplicity of the sample processing may explain the high precision of the present method (coefficient of variation 2–4%). The high reproducibility may also be related to the use of an autosampler for HPLC, which ensures high precision of the sample injections.

Samples of 25 μl were injected into a short, 3 μm ODS column. A further increase in the injection volume would increase the sensitivity of the method but the resolution would be decreased beyond that required for separation of AG from its metabolite and interfering material present in human serum.

The standard curves for AG and *N*-acetyl-AG are linear in the range of 0.1–100 μg/ml. This covers the amount of these compounds found in serum from patients on single-dose or continuous AG treatment. The sensitivity of the present method (~0.1 μg/ml) compares favorably with that of other published methods (4,5).

The HPLC method described in this paper was used in our laboratory to determine the amount of AG and *N*-acetyl-AG in serum from patients treated with AG. The amount of AG and its metabolite will be related to the effect as well as the side effects of AG, which may in turn depend on the acetylator phenotype of the patient.

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