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DETERMINATION OF PLASMA AMITRIPTYLINE BY ELECTRON-CAPTURE GAS CHROMATOGRAPHY AFTER OXIDATION TO ANTHRAQUINONE

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SUMMARY

A selective procedure is described for the determination of amitriptyline in plasma. The method involves extraction, separation of amitriptyline from its metabolites and subsequent oxidation by ceric sulphate in 5.4 *M* sulphuric acid. The oxidation product, anthraquinone, is determined by means of electron-capture gas chromatography. The metabolites were separated by a column chromatographic extraction technique. The choice of oxidation reagent, optimum conditions for the oxidation, and the electron-capture properties of anthraquinone are discussed. The method can be used to determine down to 2 ng of amitriptyline in a plasma sample; the relative standard deviation at the 50-ng level was 4.0% ($n = 8$). The levels of amitriptyline found in a series of plasma samples are compared with those obtained by gas chromatography with use of nitrogen-specific detection; the two techniques gave coincident results.

INTRODUCTION

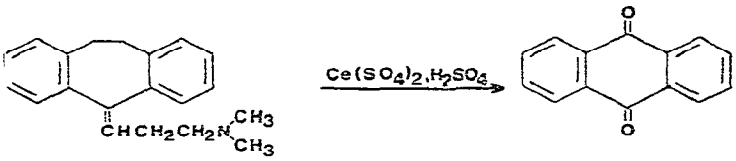
Amitriptyline is extensively used in the treatment of depressive disorders, and, owing to inter-individual differences in metabolism of the drug[1], it seems desirable to optimize therapy by means of measurement of plasma levels. Amitriptyline has been determined in plasma samples by gas chromatography (GC) with flame ionization detection[2–5], but, owing to poor sensitivity, these methods can only be used to establish the steady-state plasma level of the drug. Determination by GC with nitrogen-specific detection (GC–AFID) has been used for assaying amitriptyline after single-dose administration[6,7]. However, none of these methods takes into account the poor chromatographic properties due to the amino group in amitriptyline.

Amitriptyline has also been determined by GC after derivatization[8,9], the tertiary amine being alkylated before Hoffman degradation. The products had good chromatographic properties, but results from plasma samples were not reported.

Amitriptyline has also been determined spectrophotometrically after oxidation with potassium permanganate[10] to yield anthraquinone[11]. It occurred to us that this

oxidation could be used in combination with electron-capture GC to provide a convenient and sensitive determination. Amitriptyline is separated from its metabolites, which otherwise would be co-determined, and oxidized to anthraquinone by ceric sulphate in strongly acid medium (Fig. 1).

After completion of this work, a spectrophotometric method for the determination of high levels of amitriptyline in plasma and based on oxidation by ceric ions was published[12]; this oxidation principle was also used together with electron-capture GC[13]. However, these two methods determined amitriptyline together with its metabolites.



Amitriptyline Anthraquinone

Fig. 1. Oxidation of amitriptyline.

EXPERIMENTAL

Gas chromatography

Most of the studies were carried out with a Varian 1400 gas chromatograph. The electron-capture detector was of the ^{3}H type and was operated in the d.c. mode at 220° (corresponding to 200° at the detector foil). Two chromatographic systems were used. In this first, the glass column (150 × 0.18 cm) was packed with 3% of OV-17 on Gas-Chrom Q (80–100 mesh) and was operated at 177°; in the second, the column contained 3% of DC 560 and 0.3% of neopentyl glycol sebacate (NPGSe) on Gas-Chrom P (100–120 mesh) and was operated at 182°. The flow-rate of nitrogen carrier gas was 30 ml/min.

The temperature dependence of the electron-capture response was determined by using a Hewlett-Packard 5710 A gas chromatograph equipped with a frequency-modulated ^{63}Ni electron-capture detector. The glass column (120 × 0.2 cm) was filled with 3% of OV-17 on Gas-Chrom Q (80–100 mesh) and was operated at 220°; the carrier gas was argon plus 5% of methane and had a flow-rate of 30 ml/min.

Reagents and chemicals

Ceric sulphate solution, 0.012 *M*, in 5.4 *M* sulphuric acid, was from E. Merck (Darmstadt, G.F.R.); its concentration was determined by titration with sodium thiosulphate. The reagent was shaken twice with heptane before use.

Sulphuric acid (5.4 *M*) was of Merck Suprapur quality and was shaken once with heptane.

Cellulose (Munktell 410) was extracted with ethanol in a column to remove traces of pyridine.

The phosphate buffer solution (pH 5) was prepared by mixing 93 ml of 1 *M*

NaH_2PO_4 and 2.3 ml of 0.5 *M* Na_2HPO_4 and diluting with water to 100 ml; its ionic strength was 1.

Heptane was distilled before use.

For standardisation amitriptyline chloride was dissolved in 0.1 *M* phosphoric acid, and the solution was diluted with water to a concentration of 1.3 μg/ml; 0.05 to 1.0-ml portions of this solution were diluted with plasma to 10 ml in five different flasks, and 0.5 ml of each of these solutions were used in preparing the standard graph.

To prepare the internal-standard solution, 2-chloroamitriptyline chloride was dissolved in 0.1 *M* phosphoric acid, and the solution was diluted with water to a concentration of 1 μg/ml.

In evaluating the reaction conditions and in electron-capture-response studies, 4-bromobenzophenone was used as internal standard; it was diluted with heptane to give a concentration of 60 ng/ml.

2-Chloroamitriptyline and the metabolites of amitriptyline were kindly supplied by H. Lundbeck and Co. (Copenhagen, Denmark).

Methods

Procedure for determination of amitriptyline in plasma. Mix 0.5 ml of plasma containing not more than 100 ng of amitriptyline, 0.1 ml of the 2-chloroamitriptyline solution and 1 ml of the phosphate buffer solution of pH 5.0 carefully with 2 g of cellulose, and pack the mixture into a glass column. Elute amitriptyline and the internal standard with about 10 ml of heptane, and extract the two amines from the eluate into 1 ml of 5.4 *M* sulphuric acid.

Carefully remove the heptane by aspiration, and add 3 ml of ceric sulphate reagent to the acid solution in a test-tube. Attach an air condenser to the tube, and heat the mixture for 20 min at 100° in a glycerol bath. Then cool, add 0.1 ml of heptane, shake the mixture for 15 min and centrifuge it (5 min at 2000 rpm).

Discard the separated aqueous phase, wash the organic phase with 1 ml of 0.1 *M* sodium hydroxide, and inject 5–10 μl of the washed heptane phase into the gas chromatograph.

Procedure for determination of oxidation yield. The reaction conditions were evaluated by treating about 200 ng of the amine with oxidation reagent in the appropriate solvent. The oxidation product was extracted into heptane containing the internal standard. The yield was estimated from a standard curve prepared from known amounts of the corresponding anthraquinone and the internal standard.

Determination of electron-capture response. The minimum detectable quantity (MDQ) was determined by dilution of the pure compound with heptane. The MDQ value, as defined by Moffat and Horning[14], was obtained by comparison of the peak area obtained by injection of a known amount with that obtained by an injection of 4-bromobenzophenone. The temperature dependence of the response was evaluated by making injections of anthraquinone (in an amount about ten times the MDQ) at different temperatures. The results were expressed in terms of $cm^2/moles \times 10^{-12}$.

RESULTS AND DISCUSSION

Extraction conditions

The extraction of amitriptyline from plasma was made by a column chro-

matographic technique. The partition parameters for amitriptyline and the internal standard, 2-chloroamitriptyline, between heptane and water are given in Table I; the value of $-\log (k_d \times k_{HA})$ corresponds to the pH at which the amine has the same concentration in both phases. The pH of the stationary phase in the column was 5, which meant that amitriptyline and the internal standard appeared together with the solvent front. A 10-ml volume of heptane was collected; this was shown by a series of experiments to be satisfactory. The metabolites of amitriptyline have a much lower partition into heptane, as shown in Table I, and they were strongly retarded in the column. This will be discussed below.

TABLE I

PARTITION COEFFICIENTS OF AMITRIPTYLINE, ITS METABOLITES AND THE INTERNAL STANDARD

$k_d = A_{org}/A_{aq}$ = partition coefficient of the amine. k_{HA} = acid dissociation constant of the amine.

Compound	*log* $(k_d \times k_{HA}^*)$	*pH for 99% extraction into heptane***	*pH for <1% extraction into heptane***
Amitriptyline	−4.93	>7.0	<2.9
N-Demethylamitriptyline	−6.72	>8.8	<4.7
NN-Didemethylamitriptyline	−7.37	>9.4	<5.3
10-Hydroxyamitriptyline	−7.88	>9.9	<5.8
2-Chloroamitriptyline (internal standard)	−4.04	>6.1	<2.0

* Photometric determination.
** Equal volumes of phases.

The column step had two advantages. First, amitriptyline was separated from its metabolites, and, secondly, problems due to emulsion formation were eliminated. The yield of amitriptyline in the column step was equal to that which would have been obtained, had a simple batch extraction been used. The back-extraction of the two amines into 5.4 *M* sulphuric acid was, as shown in Table I, quantitative.

Choice of oxidation reagent

The oxidation of amitriptyline was initially studied in three oxidation systems that had already been evaluated[15,16,17].

Amitriptyline had been oxidized in alkaline potassium permanganate[10], but experiments with this reagent showed that, as previously reported[16], the yield was highly dependent on the potassium hydroxide concentration. A constant yield of 44% of anthraquinone was obtained in 0.6–3 *M* potassium hydroxide. Owing to pronounced degradation of the product in the oxidation reagent, a two-phase procedure was used, the product being continuously extracted into heptane. The simultaneous extraction of amitriptyline (as base) prolonged the reaction time, and this fact, together with poor selectivity and low precision, made this reagent unacceptable.

Two acidic oxidation reagents were also used, namely, chromic acid in 6% sulphuric acid solution in glacial acetic acid[15] and barium peroxide in 1 *M* sulphuric acid[17]. Both reagents gave yields of anthraquinone below 20%, which was insufficient for quantitative work.

Ceric sulphate in sulphuric acid was superior in the oxidation of amitryptyline; high precision and low degradation of the oxidation product, in combination with a high yield, made this the reagent of choice.

Evaluation of reaction conditions with ceric sulphate

The oxidation of amitriptyline gave anthraquinone as the main product. As it was of interest to see if dibenzosuberone was formed at any stage in the oxidation, a chromatographic system capable of differentiating between the two products was used. The yield of dibenzosuberone was less than 5% and that of anthraquinone was over 50% under optimum conditions. The following aspects of the oxidation were tested.

Temperature. Temperature had a pronounced effect on the yield. With the conditions given under Experimental, the yields were 9, 21, 56 and 54% at 40, 75, 100 and 110°, respectively. Degradation of anthraquinone occurred on prolonged reaction and was particularly pronounced when the temperature exceeded 100°.

Concentration of sulphuric acid. Besides the temperature, the oxidation was very dependent on the sulphuric acid concentration; the maximum yield was obtained in 5.3–5.8 *M* sulphuric acid.

Concentration of ceric sulphate. The yield was constant over a wide concentration range of ceric sulphate. The concentration was varied between 0.03 and 0.005 *M* without influencing the yield.

Oxidation time. The yield of anthraquinone with time is shown in Fig. 2. In the absence of an organic phase, the reaction was fast, a constant yield being obtained after 10 min. The yield remained constant for 1 h, after which a decrease was observed, probably owing to degradation. If an organic phase was present during the oxidation, the product could be prevented from degradation, but in these circumstances 1 h was needed to attain a yield of 56% (see Fig. 2).

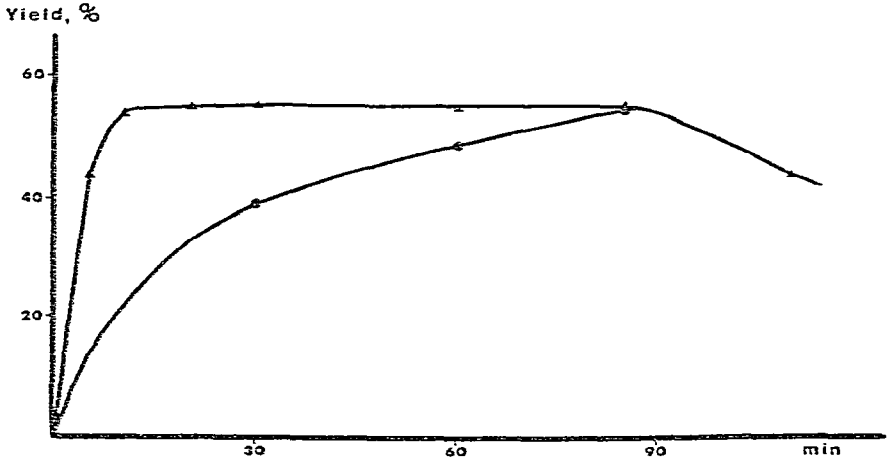


Fig. 2. Dependence of oxidation yield on time. Amitriptyline concentration, 200 ng/ml; ceric sulphate concentration, 0.012 *M*; sulphuric acid concentration, 5.4 *M*; temperature, 100°. ●—●, Organic phase present; ▲—▲, no organic phase present.

Precision of the oxidation yields. Oxidation with ceric sulphate gives clean heptane extracts for GC, and the oxidation step has high precision (the relative standard deviation was 1.6% for the oxidation of 10 identical samples).

Selectivity of the method

Amitriptyline is extensively metabolized, and the oxidation yields from some of its metabolites in ceric sulphate oxidation are shown in Table II. These yields are higher than that from amitriptyline, which means that the metabolites must be excluded before the oxidation, as they would otherwise be co-determined. Trifluoroacetic anhydride forms derivatives with primary and secondary amines, and with alcohols, but not with compounds having tertiary amino-groups. However, acylation was not possible, because 10-hydroxyamitriptyline was to some extent dehydrated and the product was co-determined in the oxidation.

TABLE II

YIELD OF ANTHRAQUINONE FROM AMITRIPTYLINE AND SOME OF ITS METABOLITES ON CERIC OXIDATION

Compound	*Oxidation yield (%)*
Amitriptyline	56.8
N-Demethylamitriptyline	70.4
NN-Didemethylamitriptyline	64.0
cis-10-Hydroxyamitriptyline	68.8
trans-10-Hydroxyamitriptyline	69.8
2-Chloroamitriptyline (internal standard)	63.9*

* 2-Chloroanthraquinone.

The metabolites could be quantitatively extracted into heptane at a pH value exceeding 10 (see Table I). However, there is a marked difference in the partition into heptane for amitriptyline and the metabolites, amitriptyline showing a partition into heptane about 70 times greater at pH 5 than that of N-demethylamitriptyline (and still higher when compared with the other metabolites). A column chromatographic extraction was therefore used, and this completely separated the metabolites from amitriptyline and from the internal standard. Experiments were carried out with a small separation column under the extraction column, but, owing to very strong adsorption of the amines in the separation column, this alternative could not be used. No interference from metabolites was noticed even on addition of very high amounts (up to 100 ng) of metabolite, provided that the percentage of stationary phase on the support was high and that the ionic strength was maintained at 1.

Gas chromatography of anthraquinone and some related compounds

Anthraquinone can be subjected to GC on moderately non-polar stationary phases, symmetrical peaks being obtained, with no indication of adsorption losses (a chromatogram is shown in Fig. 4).

As it was essential to separate anthraquinone and dibenzosuberone chromatographically in studies of the oxidation conditions, two chromatographic systems were

used. The retention of anthraquinone relative to 4-bromobenzophenone is shown in Table III. Anthraquinone and dibenzosuberone were completely resolved on the mixed stationary phase. This phase, however, causes an electron-capture response if it bleeds into the detector, so that a lower-bleeding phase (OV-17) was used in the analysis of plasma samples.

TABLE III

RELATIVE RETENTION VALUES AND ELECTRON-CAPTURE RESPONSES OF ANTHRAQUINONE AND SOME RELATED COMPOUNDS

Apparatus: Varian 1400 gas chromatograph with tritium electron-capture detector; detector-foil temperature: 205°.

Compound	*Retention relative to 4-bromobenzophenone*		*MDQ (moles/sec × 10^{-15})*
	3% DC 560 + 0.3% NPGSe column	*3% OV-17 column*	
4-Bromobenzophenone	1.00 (4.6 min)	1.00 (4.0 min)	1.2
Anthraquinone	1.50	1.95	4.7
2-Chloroanthraquinone	2.72	4.05	0.8
Dibenzosuberone	1.35	1.83	5.0

Electron-capture response of anthraquinone and its temperature dependence

Conjugated carbonyl compounds, such as benzophenones, show a high electron-capture response[18]. The minimum detectable quantity of anthraquinone with a temperature of 200° at the detector foil was 4×10^{-15} moles/sec, corresponding to about 40 pg injected under our GC conditions. As it was suspected that the response to anthraquinone could be dependent on detector temperature, injections of anthraquinone were made at different detector temperatures in two gas chromatographs with detectors working on different principles. The peak areas obtained when 10^{-12} mole of anthraquinone was injected over a wide temperature range are shown in Fig. 3. The temperature dependence was similar in the two instruments, the highest response being at high detector temperature. This indicates that anthraquinone captures electrons according to a dissociative mechanism[19]; benzophenone and acetophenone capture electrons non-dissociatively, the highest response being at low detector temperature[18].

The MDQ of dibenzosuberone was similar to that of anthraquinone, and, owing to the contribution from the chlorine atom, the response was higher for 2-chloroanthraquinone, as shown in Table III.

Choice of internal standard

In order fully to utilize the internal standard, it must have a similar structure to the drug being determined, so that it will be extracted, derivatized and chromatographed in the same manner. 2-Chloroamitriptyline was ideal in all these respects. It has a higher partition into heptane than has amitriptyline (see Table I), which means that it also appeared with the solvent front. Its yield in the ceric sulphate oxidation stage was 63.9% (see Table II) and only 20 min was required for completion of the reaction. The oxidation product had a retention of about 2 compared with anthraquinone on the stationary phases used (see Table III).

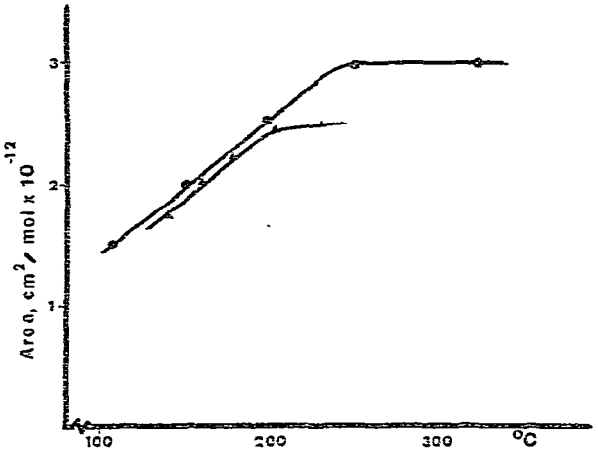


Fig. 3. Dependence of detector temperature on electron-capture response of anthraquinone. Apparatus: ●—●, Hewlett Packard 5710 A gas chromatograph with frequency-modulated ^{63}Ni electron-capture detector; ▲—▲, Varian 1400 gas chromatograph with tritium electron-capture detector operated in the d.c. mode.

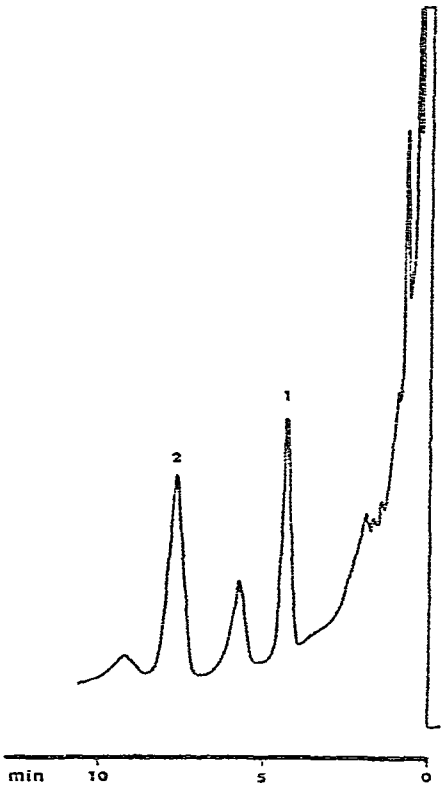


Fig. 4. Gas chromatogram of amitriptyline (91 ng/ml) from 0.5 ml of plasma 1 = Anthraquinone from amitriptyline; 2 = 2-chloroanthraquinone from internal standard.

Application to plasma samples

The method was applied to plasma samples obtained during a pharmacokinetic study in which 15 mg of amitriptyline was administered by intravenous infusion to human volunteers[20]. A chromatogram from the analysis of a plasma containing 91 ng/ml of amitriptyline is shown in Fig. 4. The lowest concentration of amitryptyline determined with this method was 2 ng/ml. The relative standard deviation for 50 ng of amitriptyline added to 1 ml of plasma was 4%.

Comparison of the method with a gas chromatographic method involving nitrogen-specific detection (GC–AFID)

The levels of amitriptyline in the plasma samples from the above-mentioned study[20] were determined by GC–AFID[6] and by the proposed oxidation method. In Fig. 5, the values of the GC–AFID method are plotted as ordinate and those from the oxidation procedure as abscissa. The regression line has a slope of 0.955 + 1.8 ng, and the regression coefficient is 0.982. These coinciding levels for plasma amitriptyline show that no interference from metabolites occurs in the oxidation procedure, owing to the employment of the column-chromatography step. The oxidation procedure is somewhat more sensitive and has a higher precision[6] (the relative standard deviations of the procedures are 4 and 7.6%, respectively).

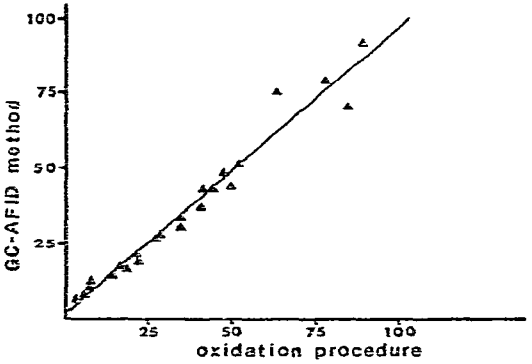


Fig. 5. Plasma concentrations obtained by the GC–AFID method (ordinate) and the oxidation procedure (abscissa).

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