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TWO-PHASE DERIVATIZATION OF AMITRIPTYLINE AND STRUCTURALLY RELATED TERTIARY AMINES FOR GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

A new derivatization technique for tertiary amines has been developed. The amine is extracted at ambient temperature after addition of sodium iodide with methylene chloride containing an aryl chloroformate ester. By use of two new reagants, 2,4-dichlorophenyl and pentafluorophenyl chloroformate, the carbamates formed have high electron-capture response. The derivatization is rapid, often completed within 5 min. Amitriptyline was determined down to 6 ng/ml directly in a spiked plasma sample. A method for selective derivatization of a tertiary amine in the presence of the corresponding secondary amine is given.

INTRODUCTION

Numerous methods for the quantitative analysis of tricyclic antidepressants *e.g.* amitriptyline (AMI) and related compounds have been published[1]. Electron-capture gas chromatography (GC) has been used to a rather limited extent, probably due to the fact that the derivatization step is often very time-consuming[2].

Derivatization of tertiary amines with trichloroethyl chloroformate (TCE) to carbamates with excellent GC performance and high electron-capture response has been reported previously[2,3].

Most of the methods for trace analysis of compounds in complex aqueous matrices, *e.g.* plasma, are laborious and involve several extraction steps before the final assay[1]. Furthermore, precautions against adsorption losses during the extractions are often necessary but not always successful[4,5].

A technique for rapid quantitative determination of tertiary amines in biological material after derivatization with chloroformate esters has recently been reported[6]. The plasma sample is extracted with an organic solvent containing a chloroformate ester. Extraction and derivatization therefore occur in the same procedural step. In such an approach, adsorption losses should be of minor importance. Furthermore, the combined extraction and derivatization procedure can be performed at neutral or slightly acidic pH which is advantageous when dealing with compounds

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unstable at high pH, *e.g.* phenothiazines[5]. The procedure given in ref. 6 could, however, be applied only to compounds containing benzylic or allylic groups at the nitrogen atom.

This study presents a further development of the derivatization technique, which makes the method applicable also to N-methyl substituted tertiary amines. Reaction conditions for determination of amitriptyline in presence of plasma are given.

EXPERIMENTAL

Apparatus

Gas chromatography. A Pye Unicam gas chromatograph Series 106 equipped with a flame ionization detector was used for evaluation of reaction conditions.

Low concentration analysis was performed with a Hewlett-Packard 5710 A gas chromatograph equipped with a ^{63}Ni electron-capture detector. Argon with 5% of methane was used as carrier gas (flow-rate 30 ml/min).

The glass columns (120 × 0.18 cm I.D.) were filled with 3% OV-17 on Gas-Chrom Q, 100–120 mesh, and operated at 310°C for analysis of AMI as the 2,4-dichlorophenyl carbamate.

Mass spectrometry. An LKB 9000 mass spectrometer was used with an ionization energy of 70 eV. The compounds were introduced by GC using a column as above.

Chemicals and reagents

Methylene chloride and trichloroethyl chloroformate (TCE) (Ega-Chemie, G.F.R.) were distilled before use, the latter in vacuo. Toluene Uvasol^{R} (E. Merck, G.F.R.) was used as recieved.

2,4-Dichlorophenyl chloroformate (DCP) and pentafluorophenyl chloroformate (PFP) were synthesized from 2,4-dichlorophenol (Fluka, Switzerland) and pentafluorophenol (Fluka), respectively, by treatment with 20% phosgene in toluene (Fluka) according to the general procedure described in ref. 7. Assay gave a content of 98% for DCP and 96% for PFP. Alcoholic alkali was prepared by dissolving 2.8 g of potassium hydroxide in 75 g of methanol and 25 g of distilled water. All other chemicals were of analytical grade. The identity of all derivatives was confirmed by mass spectral analysis.

Methods

Evaluation of reaction conditions. The amine ($5 \cdot 10^{-5} M$) was dissolved in phosphate buffer pH 6.0 ($\mu = 0.2$, unless otherwise stated) containing sodium iodide. The appropriate chloroformate ester was dissolved in methylene chloride containing the internal standard. Equal volumes (2.00 ml) of aqueous and organic phases were shaken in centrifuge tubes at 20°C. The reaction was quenched by addition of 5 ml of 0.2 *M* sulphuric acid.

The peak area ratio to the internal standard derivative was calculated. The absolute yield was determined by a comparision with peak area ratio obtained with a reference sample containing known amounts of the derivatives.

The internal standards were: desmethyl-2-chloroimipramine for AMI, nortrip-

tyline (NTP) for 2-chloroimipramine (CIM) and 4-phenyl-1,2, 3,6-tetrahydropyridine for pethidine.

Determination of amitriptyline in spiked plasma samples. Plasma (1.0 ml) in a small centrifuge tube (8 ml) is mixed with 1.0 ml of a phosphate buffer solution pH 6.0 ($\mu = 0.2$) containing AMI (6–85 ng/ml), CIM (35 ng/ml) and sodium iodide (1.0 *M*) and 2.0 ml $3 \cdot 10^{-3}$ *M* solution of DCP in methylene chloride. The mixture is shaken for 10 min at 20°C.

The tube is centrifuged for 2 min, and the organic phase is transferred to another extraction tube containing 0.5 ml of toluene. The organic phase is reduced to *ca.* 0.5 ml by evaporation. Alcoholic alkali (0.5 ml) is added and a homogenous solution is obtained. After 5 min, 2 ml of 2 *M* sodium hydroxide is added and the tube vigorously shaken for 30 sec. A few microlitres of the organic phase is taken to analysis and quantitated by peak area measurements.

Determination of amitriptyline in presence of nortriptyline. A 2.0-ml volume of a phosphate buffer solution, pH 6.0, containing AMI (90 ng/ml), CIM (102 ng/ml) and NTP (1100 ng/ml) is mixed with 2.0 ml of a $4 \cdot 10^{-3}$ *M* solution of TCE in methylene chloride. The mixture is shaken for 5 min, then 0.5 ml of 2.5 *M* sodium iodide and 0.5 ml of $1.5 \cdot 10^{-2}$ *M* DCP in methylene chloride are added and the tubes are shaken for 10 min. The organic phase is treated as above.

RESULTS AND DISCUSSION

Reaction principles in two-phase systems

The aqueous phase containing the amine is extracted with an organic solvent, *e.g.* methylene chloride containing the chloroformate ester. In the organic phase, the amine base reacts with the reagent initially to an intermediate chloride ion pair[8]. The intermediate ion is distributed between the two phases and reacts with nucleophiles present, *e.g.* halide ions, to give a carbamate and an organic chloride (Fig. 1, path I). The intermediate ion can also react with water in both phases, giving the original amine (path II)[6,8,9]. A further reaction pathway involves a nucleophilic attack on the alkoxy part of the intermediate ion. In such a reaction, an alkyl halide and the original amine would be formed (path III)[10].

N-Methyl tertiary amines do not react with alkyl chloroformate esters such as TCE at ambient temperature to give carbamates[2] as is the case for benzyl- or allyl-substituted tertiary amines[6]. This might be due to extensive reactions according to both reaction paths II and III. A reaction according to path III should be of less

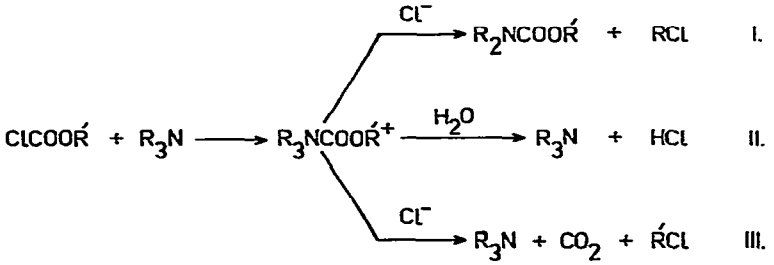

Fig. 1. Reaction pathways for the intermediate ion. R_3N, tertiary amine; ClCOOŔ, chloroformate ester; R_3NCOOR^+, intermediate ion; R_2NCOOR, carbamate.

importance if an aryl chloroformate ester is used[10]. However, the hydrolysis reaction is not eliminated and this leads to consumption of the reagent with a concomitant liberation of hydrogen chloride. This will give a decrease in pH, which in turn will increase the ion-pair distribution of the amine to the organic phase and, as a result, lower the reaction rate for carbamate formation.

If an aryl chloroformate ester, such as DCP or PFP, is used in combination with a strong nucleophilic agent like iodide ion[6], reaction pathways II and III are strongly supressed and rapid carbamate formation occurs even at room temperature.

Reaction conditions

The influence of iodide ion in the aqueous phase on reaction yield is shown in Table I. The reactions were followed up to 30 min. Both AMI and CIM gave a constant yield within 5 min. The low yield obtained at an iodide concentration of 0.05 *M* is ascribed to a higher extent of hydrolysis, which indirectly gives rise to a complete consumption of the reagent (DCP). Essentially the same reaction behavior was found for other tertiary amines, such as promazine and imipramine. When pentafluorophenyl chloroformate (PFP) was used as reagent the effect of hydrolysis was even more pronounced. However, with an increased buffer capacity ($\mu = 0.4$), 0.5 *M* sodium iodide and a tenfold increase of the PFP concentration ($3 \cdot 10^{-2}$ *M*), the yield, *e.g.* for CIM, was quantitative within 5 min.

TABLE I

INFLUENCE OF IODIDE ION CONCENTRATION ON THE YIELD OF 2,4-DICHLOROPHENYL CARBAMATES

Amine	*Yield (%)*	
	Iodide ion concentration (M)	
	0.05	*0.5*
Amitriptyline	29	99
2-Chloroimipramine	52	101
Pethidine	40*	100*

* Reaction times, 25 min; aqueous phase, phosphate buffer ($\mu = 0.2$) pH 6.0; organic phase, $3 \cdot 10^{-3}$ *M* 2,4-dichlorophenyl chloroformate in methylene chloride.

The choice of pH is a key point since the amine only reacts as base. At a low pH, the reaction rate is reduced since the distribution ratio as base is low owing to a high distribution ratio as ion pair with iodide. On increase of pH, the distribution ratio of the amine as base increases but there is also a rapid increase in the hydrolysis rate of the intermediate ion[6].

The influence of the organic solvent on the reaction rate is mainly due to its effect on the distribution of the amine and the intermediate ion pair[6]. Solvents promoting high ion-pair distribution of the intermediate ion to the organic phase are preferred since the hydrolysis rate in the aqueous phase is high. However, hydrolysis can also occur in the organic phase, which is why solvents with low ability to dissolve

TABLE II

ELECTRON-CAPTURE RESPONSE AHD RELATIVE RETENTION FOR 2,4-DICHLOROPHENYL AND PENTAFLUOROPHENYL CARBAMATES

Detector temperature: 325°C. t_R = Retention time.

Amine	*Minimum detectable concentration* ($mol \cdot sec^{-1} \cdot 10^{16}$)		$t_{R(DCP)} \cdot t^{-1}_{R(PFP)}$
	DCP	*PFP*	
Amitriptyline	5	4	5.7
2-Chloroimipramine	4	–	4.8
Pethidine	3	2	6.6

water should be used. Methylene chloride, which fulfills these requirements. is therefore a suitable solvent.

Table II shows the elctron-capture response for some of the derivatives determined at optimum detector temperature. In all cases it was found that the response increased slightly with increasing detector temperature, levelling off at about 300°C. The choice of reagent is not very critical with respect to the magnitude of the electron-capture response.

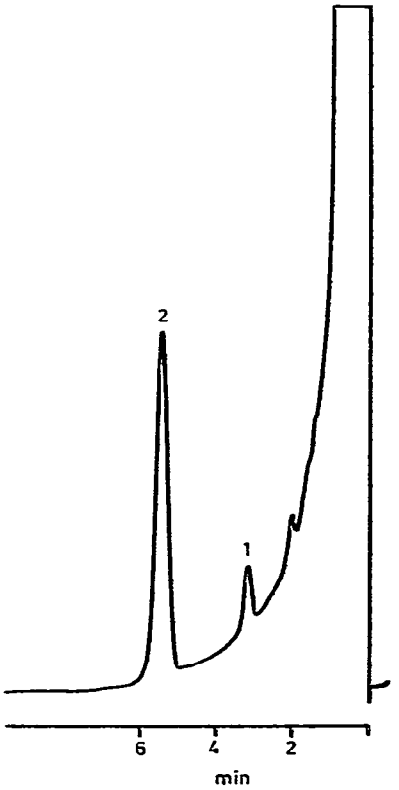

Fig. 2. Gas chromatogram obtained after a two-phase derivatization of amitriptyline (6 ng/ml) in a spiked plasma sample. Peaks: 1 = DCP-amitriptyline; 2 = DCP-2-chloroimipramine.

Analysis of amitriptyline in the presence of plasma

The two-phase reaction can easily be performed with spiked plasma samples. DCP is preferred to PFP as reagent mainly owing to the lower concentration necessary for derivative formation.

Methanolic alkali is added to remove excess of the reagent and other by products that disturb the GC analysis[3,11].

The peak area ratios were the same as those obtained from compounds dissolved in phosphate buffer solutions, indicating a quantitative yield of both AMI and CIM derivatives. The time for quantitative yield of the derivatives was not influenced by plasma.

The standard curves were rectilinear passing the origin in all cases. The lowest concentration determined was 6 ng/ml (Fig. 2).

Selectivity

Chloroformate esters react fast and quantitatively with secondary amines[12]. This means that if a plasma sample containing AMI and its main metabolite NTP (N-desmethylamitriptyline) is treated according to the method for AMI the same derivative is formed from both the tertiary and the secondary amine. However, if the sample is first extracted with a solvent containing an alkyl chloroformate ester, only the secondary amine forms a carbamate. When this reaction is completed, sodium iodide and an aryl chloroformate ester are added and the tertiary amine is derivatized. This principle was tested with AMI and NTP using trichloroethyl chloroformate and 2,4-dichlorophenyl chloroformate as reagents. The peak area ratios obtained did not differ from those obtained when NTP was absent. With an appropriate combination of the two chloroformate esters it should be possible to quantitate both the tertiary and secondary amine within the same chromatographic run.

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