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DETERMINATION OF CARBOXYLIC ACIDS IN THE PICOMOLE RANGE AFTER DERIVATIZATION WITH PENTAFLUOROBENZYL BROMIDE AND ELECTRON CAPTURE GAS CHROMATOGRAPHY

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SUMMARY

A method is presented for the determination of picomole quantities of carboxylic acids by gas chromatography in combination with electron capture detection. The acids are extracted from aqueous media into dichloromethane by ion-pair extraction with tetrapentylammonium ions, and derivatized as their pentafluorobenzyl esters. These derivatives have good chromatographic properties with minimum detectable amounts of *ca.* 0.15 pg. 250 pg or greater quantities of the acids can be used. Recoveries are *ca.* 90% with a precision of *ca.* 6% at the 10-ng level.

INTRODUCTION

A large number of therapeutic drugs comprise esters of carboxylic acids. Drugs of this type include potent anticholinergics such as atropine and oxyphenonium bromide, analgesics such as pethidine and tranquillizers such as benactyzine. These highly active drugs are usually administered in small quantities and, accordingly, the levels found in biological fluids and tissues are in the ng/ml range. Therefore, a highly sensitive and reliable assay in the picomole range is necessary in order to study these drugs in biological samples.

Unfortunately, direct analysis of the esters poses several problems, particularly with regard to stability and sensitivity when techniques such as gas–liquid chromatography (GLC) and high-performance liquid chromatography are used. In an effort to circumvent these problems we have investigated whether hydrolysis of the esters, followed by analysis of a suitable derivative of the acid moiety, would be a useful alternative for GLC.

Pentafluorobenzyl (PFB) derivatives of carboxylic acids are known to exhibit high sensitivities in electron capture detectors (ECDs)[1–6], and several analytical procedures have been proposed to convert carboxylic acids into their respective PFB derivatives[1–7]. However, all these methods use rather high concentrations of acids and reagents, and cannot be adapted easily to carboxylic acid quantities in the picomole range. The major problems encountered are: (i) long reaction times and non-

quantitative yields; (ii) interference from excess of PFB–Br which cannot be handled by the ECD; (iii) contamination of samples and glassware by acidic components of biological nature such as fatty acids. A recent paper by Gyllenhaal *et al.*[6] has re-emphasized these difficulties.

The present paper reports a solution to these problems and describes an assay method for carboxylic acids in the picomole range which is based on ion-pair extraction in dichloromethane with tetrapentylammonium as counter ion, derivatization in dichloromethane with small amounts of PFB-Br and GLC–ECD.

EXPERIMENTAL

Apparatus

A Hewlett-Packard Model 5830 gas chromatograph equipped with a 15-mCi ^{63}Ni pulse-frequency-modulated ECD was used. The glass column (180 cm × 0.2 cm I.D.) was coated with HMDS and packed with 3% OV-17 on Chromosorb W HP (80–100 mesh). The carrier gas was argon–methane (95:5; flow-rate, 30 ml/min) which had been dried over molecular sieves (3 Å). Temperatures: injector, 300°; column, 235°; detector, 300°.

Glassware was cleaned by standing overnight in a mixture of 80 ml of hydrogen peroxide (36%), 300 ml of hydrochloric acid (36%) and 120 ml of distilled water, then rinsed with distilled water and dried at 105°. The PTFE-lined screw-caps of the extraction and derivatization tubes were cleaned in a solution of 2% Extran® MA 01 (Merck, Darmstadt, G.F.R.) by heating to 95°, cooling and standing overnight. Rinsing was as described above.

Chemicals and reagents

α-Cyclohexyl-α-phenylglycolic acid (CHPGA) was kindly supplied by Ciba-Geigy (Basle, Switzerland). Tetrapentylammonium iodide (TPeA–I) was from Eastman-Kodak (Rochester, N.Y., U.S.A.); pentafluorobenzyl bromide (PFB–Br), α-cyclohexyl-α-phenylacetic acid (CHPAA) and α,α-diphenylacetic acid (DPAA) were from Aldrich-Europe (Beerse, Belgium). α,α-Diphenylglycolic acid (benzilic acid, BA) and β-hydroxy-α-phenyl-proprionic acid (tropic acid, TA), as well as all of the other chemicals and solvents (analytical grades), were from Merck.

Dichloromethane (DCM) was purified by passage down a column of molecular sieves (3 Å), followed by a column containing silica gel 60 (extra pure for column chromatography; 70–230 mesh) and finally by distillation. 0.2% ethanol (99%) was added to the distillate (40°).

Tetrapentylammonium solution ($2 \cdot 10^{-3}$ *M*; pH = 7.5) was prepared as follows. 2.13 g of TPeA-I and 0.637 g of silver oxide were added to 30 ml of distilled water and the mixture was allowed to react for 1 h in an ultrasonic water-bath at room temperature. The solution was filtered and the filtrate was extracted twice with equal volumes of DCM. The aqueous layer was neutralized with phosphoric acid (0.1 *M*) to pH 7.5 and again extracted twice with equal volumes of DCM. The TPeA concentration in the remaining aqueous phase was determined by the method of Gustavii and Schill[8], and the solution was diluted to the required concentration.

A solution of 0.1% pentafluorobenzyl bromide was prepared by adding 10 μl of PFB–Br to 10 ml of purified DCM.

Derivatization on a preparative scale

PFB derivatives of the acids were prepared according to Kawahara[1] using 0.002 mole of the respective acids. BA–PFB and CHPGA–PFB were obtained as colourless oily liquids, whereas CHPAA–PFB, DPAA–PFB and TA–PFB were obtained as white crystalline materials. The derivatives were dissolved in *n*-heptane–ethyl acetate (98:2) to give solutions containing 1.0, 2.0, 5.0, 10.0, 20.0, 50.0, 100 and 200 picomole of each acid per 5 μl. For each acid, a standard response curve was obtained by injection of 5 μl of these solutions in to the gas chromatograph, using one of the other derivatized acids as internal standard at a fixed concentration of 20.0 picomole per 5 μl.

Extraction and derivatization in the picomole range

Standard solutions of 0.0, 0.25, 0.50, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0 and 100 ng of each acid were prepared in 50 μl of 10^{-3} *M* KOH, containing a suitable acid as internal standard at a fixed concentration of 10 ng per 50 μl. For example, BA was used as internal standard for the CHPGA solutions. 50 μl of the standard solutions were pipetted into glass tubes having PTFE-lined screw-caps (Sovirel® 13). 400 μl of TPeA solution ($2 \cdot 10^{-3}$ *M*), 50 μl of NaOH (1 *M*) and 100 μl of NaH_2PO_4 (1 *M*) were added. The mixture (Ph 7.1) was extracted for 30 sec with 1.0 ml of purified DCM on a Vortex mixer, cooled in ice for 5 min and centrifuged (*ca.* 4800 *g*) at *ca.* 0° for 5 min. The aqueous phase was siphoned off, and 500 μl of the DCM phase were transferred to a 1-ml Reacti-Vial (Pierce, Rockford, Ill., U.S.A.) and taken to dryness under a gentle stream of nitrogen. 50 μl of PFB–Br (0.1%) were added, and the tube was sealed, heated at 65° for 30 min in a blockheater (Supelco, Bellefonte, Pa., U.S.A.) and cooled for 5 min at room temperature. The residue was taken to dryness under a gentle stream of nitrogen and then redissolved in 50 μl of *n*-heptane–ethyl acetate (98:2). 5 μl of this solution were injected into the gas chromatograph. The peak areas were obtained by electronic integration. The reproducibility and recovery were tested with 10.0 ng of CHPGA and 10.0 ng BA per 50 μl.

RESULTS AND DISCUSSION

The synthesis of CHPGA–PFB, BA–PFB, CHPAA–PFB, DPAA–PFB and TA–PFB, on the preparative scale according to Kawahara[1], gave yields of *ca.* 80%, but attempts to apply the same principles to the nanogram range were unsuccessful. Recoveries were *ca.* 30–40% and could not be improved by the addition of a crown ether as recommended by Durst *et al.*[9]. The use of other organic solvents or changes in the amounts of alkali did not give better results, which is in agreement with observations of Wickramasinghe *et al.*[2]. Brändström and Junggren[10] emphasized the increased reactivity of anions when dissolved in solvents having poor solvating properties, such as chlorinated hydrocarbons. This principle has been applied by Ehrsson[7] in an extractive alkylation procedure. Although excellent results were reported, the method was developed only for rather high quantities of acids ($2 \cdot 10^{-6}$ mole) and PFB–Br ($6.8 \cdot 10^{-5}$ mole) and for use with a flame ionization detector. These factors render the approach unsuitable for nanogram quantities which require electron capture detection and, accordingly, adequate removal of excess of PFB–Br. More recently, Gyllenhaal *et al.*[6], using the same approach, succeeded in removing excess

of PFB–Br by coupling it with a alkylaminophenol and subsequent extraction of the product into an acidic aqueous phase. However, these workers were unable to overcome the high levels (200 ng per sample) of contamination by palmitic acid.

In our procedure we chose a single ion-pair extraction into dichloromethane using $TPeA^+$ as counter ion at a suitable pH. Yet, when trying to scale down this approach to nanogram amounts it soon became apparent that for such low quantities knowledge of the various extraction parameters involved was a prerequisite.

It was found that association of the acids with TPeA in the aqueous phase could be an important side reaction, which had an adverse effect on the extraction of the acids in the organic phase. With tetrabutylammonium as counter ions, this association was even more pronounced. For example, for CHPGA ($1.8 \cdot 10^{-6}$ *M*) and TPeA ($2.1 \cdot 10^{-4}$–$2.1 \cdot 10^{-6}$ *M*), the extraction constant E and the association constant K_{ass} were determined essentially according to Modin and Schill[11] and Lagerström[12] respectively and were found to be $E = 1.2 \cdot 10^5$ and $K_{ass} = 2.1 \cdot 10^4$. Based on these values, an optimum extraction procedure was developed as described in the Experimental section. The pH of the aqueous phase should be at least 7 to give adequate dissociation of the acids so that ion pairs can be formed. However, at pH > 8, fatty acids, which may be present as contaminants, or as endogenous components in biological samples, begin to interfere.

The effect of the association phenomena on the recovery is shown in the following example: for a concentration of 10 ng of CHPGA in 600 μl of an aqueous solution containing $1.3 \cdot 10^{-3}$ *M* TPeA, the conditional extraction constant E^* is $4.1 \cdot 10^3$, which gives a distribution coefficient $D = 5.5$. Thus, the recovery P for a 0.6:1 extraction is 90.2%.

After the extraction of the acids, derivatization is carried out in 50 μl of dichloromethane to which 0.1% PFB–Br has been added. It is very important to adhere to this low concentration of PFB–Br. Higher amounts of this reagent will reduce the reaction time and temperature needed for complete derivatization, but will also drastically increase, with concomitant loss of PFB esters, the time needed to take the sample to dryness due to the low volatility of PFB–Br (b.p. 174°). The present choice of 0.1% PFB–Br provides a suitable compromise: the reaction time can be kept at 30 min at 65°, whereas the excess of reagent can still be removed rather easily by evaporation under nitrogen, possibly under azeotropic conditions, without the need for additional clean-up procedures. The PFB derivatives of the acids show excellent GLC behaviour (Fig. 1). The retention times for the PFB esters are listed in Table I. With TA–PFB some peak tailing was observed and this component also showed a minor (< 1%) second peak due to α-phenylacrylic acid–PFB. The latter compound may be formed by decomposition of TA during the derivatization or in the injection port, or from α-phenylacrylic acid present as an impurity in the starting material. The structures of the derivatives were confirmed by GLC–mass spectrometry. No losses occurred in the gas chromatograph even after an injection of less than 1 pg of an acid. The derivatives were stable for at least 6 months at room temperature when stored in *n*-heptane–ethyl acetate (98:2).

Minimum detectable amounts (MDAs) were determined by injecting 0.7 pg of the particular PFB derivatives and are listed in Table I. For all of the derivatives, linear calibration curves were obtained over the range from the MDA up to 5 ng of the acid.

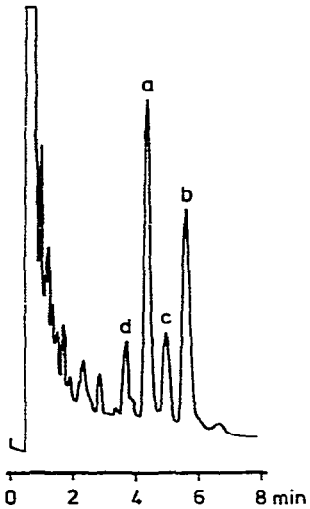

Fig. 1. Gas chromatogram of CHPGA at the 10-ng level with 10 ng of BA as internal standard. Peaks: a = CHPGA–PFB; b = BA–PFB; c = palmitic acid–PFB (contaminant); d = unknown contaminant from the septum of the derivatization tubes.

TABLE I

RETENTION TIMES AND ECD RESPONSE OF SOME CARBOXYLIC ACIDS AS THEIR PFB DERIVATIVES

Compound	*Retention time (min)*	*Minimum detectable amount**	
		10^{-17} mole/sec	*pg***
BA–PFB	5.95	11.7	0.18
CHPAA–PFB	3.53	3.0	0.05
CHPGA–PFB	4.61	6.9	0.11
DPAA–PFB	4.75	3.9	0.06
TA–PFB	2.05	8.6	0.09

* Defined as a signal three times the background noise level, at a retention time of 3 min for a column with 3800 theoretical plates[13].

** Expressed in terms of the acids.

Fig. 2 represents the standard response curve for the determination of CHPGA using BA as internal standard, obtained by fitting the data to the equation $y = A \cdot x^b$. This gave a correlation coefficient of 0.9997 for a value of b of 1.014. It should be noted that this response curve may also be used for quantities lower than 1 ng, down to 0.25 ng, but for such cases a smaller amount of internal standard is preferred.

The overall recoveries of the procedure are given in Table II. Bearing in mind that the maximum recovery of the extraction procedure is *ca.* 90%, it can be concluded that the yield of the derivatization step at the nanogram level must be *ca.* 100%. The reproducibility of the determination of 10 ng of CHPGA using 10 ng of BA as internal standard was found to have a coefficient of variation of 6.3% ($n = 8$).

It is emphasized that an assay procedure for nanogram quantities is highly

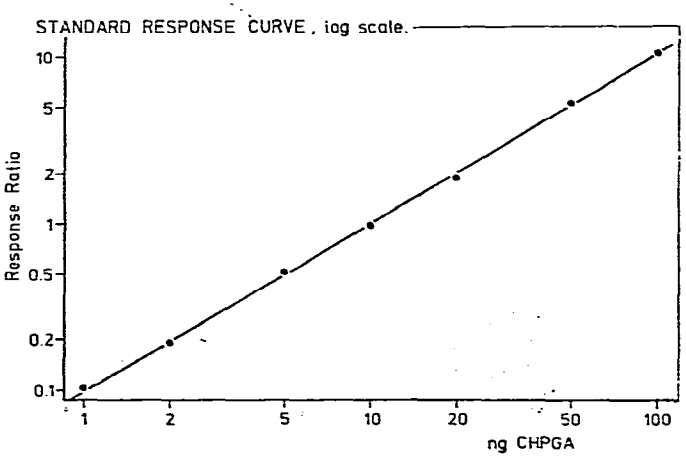

Fig. 2. Standard response curve for the determination of CHPGA with 10 ng of BA as internal standard.

TABLE II

RECOVERIES FOR THE DETERMINATION OF CHPGA AND BA AT THE 10-ng LEVEL (SEE EXPERIMENTAL SECTION)

Acid	*Mean recovery (%)*	*Variation coefficient (%)*	*No. of determinations*
CHPGA	88.7	6.3	8
BA	87.8	3.6	8

sensitive to interference from contaminants. In the present procedure, contamination by fatty acids was a major problem, particularly that by palmitic acid. The PFB derivative of palmitic acid has a retention time close to that of the CHPGA and BA derivatives (see Fig. 2); moreover this contaminant was found to be present on high concentrations relative to the acids under investigation. Therefore, special procedures were necessary for cleaning all of the glassware. Contact of the skin with the glassware should be kept to a minimum and must be strictly avoided with glassware that has a wet outer surface since fatty acid contaminants may creep from the outside to the inside of the glassware.

Fatty acids and other contaminants were also found to be present in the TPeA solutions, which required purification by ion-pair extraction. The dichloromethane also needed to be purified. In the latter procedure, the small amounts of ethanol which are present as stabilizer are removed, and thus 0.2% ethanol must be added to the purified DCM in order to stabilize it in the derivatization procedures. By strictly observing these precautions we could keep our background levels of palmitic acid to less than 2 ng per sample.

Another critical factor is the phase separation after the extraction. Owing to the relatively high surface tension of TPeA, micelle formation may occur which, in turn, increases the interference from fatty acids. This problem can be avoided by cooling to 0° prior to, and during, the centrifugation.

It should be noted that the present method was developed especially for car-

boxylic acids that are present in a number of important therapeutic drugs. Interference from other acidic components can be avoided by careful selection of three variables: the pH of the final extraction step, the nature of the cationic ion and the concentration of the latter. However, these three variables can also used in the determination of other acids; for example, fatty acids can be analyzed with the same procedure, provided that the pH of the final extraction is at least 12.

An advantage of the present method is that it makes use of a single ion-pair extraction step, which considerably reduces the interference from other acidic components, without affecting the recovery. This is demonstrated by the very low limit of detection and determination that is obtained. Other approaches, such as extractive alkylation[6] and crown ether-catalyzed derivatization[14] seem to be less selective due to the much higher backgrounds of interfering components.

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