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Note

Gas chromatographic determination of clonazepam

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Clonazepam is an anti-epileptic drug for which therapeutic monitoring of plasma levels is frequently requested. Because the therapeutic concentration is very low (30—60 ng/ml) [1] drug analysis has been limited to gas chromatography (GC) with electron-capture detection. Clonazepam has been determined without derivatisation [2], as the ethylated derivative [3], and, most commonly by hydrolysis in acid to 2-amino-2′-chloro-5-nitrobenzophenone (ACNB) [4]. ACNB has the advantage of being a stable derivative which gives excellent peak profiles and quantitation. The main disadvantages of the latter method are the prolonged acid hydrolysis step requiring a minimum of 20 min, and difficulty in separating the product from plasma constituents and contaminants by GC. This paper reports a study to improve the yield and rate of hydrolysis of clonazepam to ACNB and other minor improvements to the assay.

MATERIALS AND METHODS

Chemicals

Borate buffer, pH 9.0 [4] (contaminants if present may be removed by extracting with toluene) and 2 *M* sodium hydroxide were used. Concentrated hydrochloric and sulphuric acids from J.T. Baker (Phillipsburg, NJ, U.S.A.) were diluted as required. It is essential that the concentrated hydrochloric acid be extracted with toluene. The solvents diethyl ether, heptane, methanol obtained from May and Baker, Dagenham, Great Britain and toluene from Baker were distilled before use.

The standard, clonazepam (Roche, Dee Why, Australia) was 0.015 g in 100 ml of ethanol. Dilute 100 μl to 40 ml (375 ng/ml) with acetone—heptane (1:4, v/v) [4]. The internal standard was desmethylflunitrazepam (Roche) 700 ng/ml in acetone—heptane (1:4, v/v).

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Gas chromatography

A Pye-Unicam 204 equipped with a ^{63}Ni electron-capture detector was used. The temperature settings were: detector 350°C; column 255°C and injector 300°C. The flow-rate was 120 ml/min of oxygen-free nitrogen. No purge gas was used. Glass columns (1.5 m × 4 mm I.D.) were packed with 3% OV-17 or 3% OV-225 on 100—120 mesh Gas-Chrom Q (Applied Science Labs., State College PA, U.S.A.). For the routine method a column packed with a mixture of equal parts by weight of these two phases is used.

Standard method

Internal standard (50 μl), borate buffer (1 ml), and diethyl ether—heptane (40:60, v/v, 2 ml) were added to 0.5 ml of plasma. After shaking and centrifuging, the upper phase was back-extracted into concentrated hydrochloric acid (0.5 ml) in Kimax screw-cap tubes.

After centrifuging, the upper phase was aspirated off and methanol (1.5 ml) and diethyl ether (0.5 ml) were added. The tubes were capped and heated in a boiling water bath for 10 min, then cooled and 3 ml of 2 *M* sodium hydroxide and 2 ml of diethyl ether added. After extraction the ether phase was recovered, evaporated in a stream of air and the residue taken up in 50 μl of toluene. An aliquot of 1—2 μl was injected into the chromatograph.

Hydrolysis experiments

Experiments were done to test the effect of various acids with or without the addition of solvents on the yield of ACNB from clonazapam. Aliquots of clonazepam and desmethylflunitrazepam standards were placed in tubes and the solvent evaporated off. The required mixture of acid and solvent was then added and the tubes heated in a boiling water bath for the required time. The tubes were then cooled as quickly as possible and the hydrolysate prepared for GC as previously described. Yield of ACNB was assessed solely in terms of peak height of replicate 1-μl injections.

RESULTS

The effect of various acids and solvent mixtures on the conversion of clonazepam to ACNB when hydrolysis time is restricted to 15 min or less, is depicted in Fig. 1. Hydrolysis with concentrated hydrochloric acid gives the lowest yield of ACNB. Yield is greatly increased by the addition of methanol to the concentrated hydrochloric acid, the maximum yield being obtained with a ratio of concentrated hydrochloric acid—methanol of 0.5:1.5, v/v.

Greater amounts of methanol depress the amount of ACNB formed. Addition of diethyl ether to concentrated hydrochloric acid—methanol (0.5:1.5) further increases the yield at 10 min and is optimal with the mixture concentrated hydrochloric acid—methanol—ether (0.5:1.5:0.5). Further small increases in the proportion of diethyl ether has no additional effect on the yield, while excess diethyl ether results in phase separation. Addition of diethyl ether to concentrated hydrochloric acid without methanol depresses the yield.

With the mixture concentrated hydrochloric acid—methanol—diethyl ether (0.5:1.5:0.5), the maximum yield is obtained by boiling the tubes for 10 min.

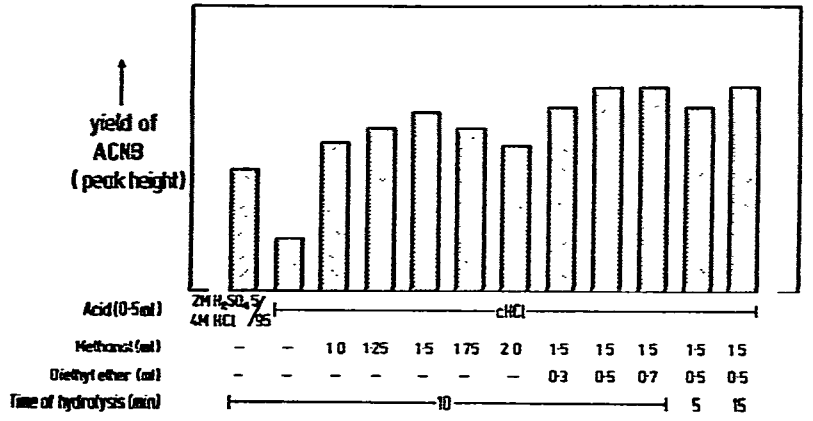

Fig. 1. Yield of ACNB from the hydrolysis of clonazepam at 100°C in various acid and solvent conditions.

After 5 min of heating the yield is approximately 90% of maximum and heating for 15 min gives a yield identical to that obtained at 10 min. Compared to the method presented in this paper the widely used mixture of 2 *M* sulphuric acid—4 *M* hydrochloric acid (5:95) produces less ACNB (93%) and requires a much longer period of heating (30 min).

The within-run coefficient of variation (C.V.) at a clonazepam concentration of 39.2 ng/ml for this method is 3.7% and the between-run C.V. is 6.7%. The detector response is linear over a plasma clonazepam concentration of 5—100 ng/ml and the minimum detectable concentration of clonazepam is less than 2 ng/ml. Typical chromatograms are shown in Fig. 2.

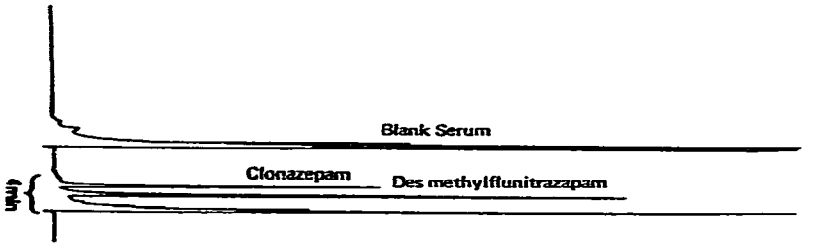

Fig. 2. Chromatograms, after extraction and hydrolysis of a serum containing clonazepam (40 ng/ml) and desmethylflunitrazepam, and of a blank serum.

DISCUSSION

Hydrolysis reactions are commonly solvent dependent. These experiments show that clonazepam can be hydrolysed to ACNB with optimum yield, within 10 min by using a mixture of concentrated hydrochloric acid—methanol—diethyl ether (0.5:1.5:0.5). Not only is the assay time shortened but yields are improved. Also side-reactions and contaminants encountered in other methods we have tried have been minimized. Experiments not reported here have shown that desmethylflunitrazepam and nitrazepam are hydrolysed faster than clonaz-

epam. Nitrazepam can be used as an alternative internal standard to desmethylflunitrazepam.

Diethyl ether is commonly used as the extraction solvent for clonazepam. In these studies it was found to co-extract unwanted plasma constituents. This was overcome by using heptane—diethyl ether (60:40, v/v). Failure to use freshly redistilled diethyl ether gives rise to side-reactions which affect quantitation. Similarly, concentrated hydrochloric acid also may contain products which give side-reactions [4]. These may be either removed or destroyed by washing the acid with toluene. Occasionally concentrated hydrochloric acid so treated discolours on standing, but this has no discernable effect on the method. The extraction of concentrated hydrochloric acid was a crucial step in obtaining a clean chromatogram.

On OV-17 co-extracted plasma constituents are not well separated from the internal standard while with OV-225, analysis time is excessive. A liquid phase of intermediate polarity would be ideal but we have found a mixture of equal parts by weight of the two phases suitable for routine work.

No other anticonvulsants are known to interfere with the assay. However, nitrazepam is only partly resolved from the internal standard on the mixed phase column at 255°C. In such a case OV-225 would be the phase of choice.

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