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DETERMINATION OF TOCAINIDE IN HUMAN PLASMA BY GAS CHROMATOGRAPHY WITH NITROGEN-SELECTIVE DETECTION AFTER SCHIFF BASE FORMATION

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SUMMARY

Tocainide is a primary amine with antiarrythmic properties derived from lidocaine. For biopharmaceutical and pharmacokinetic purposes an assay was developed that made use of Schiff base formation with methyl isobutyl ketone and gas chromatography with nitrogen-selective detection. The derivatization procedure was performed at 85°C for 10 min, although a longer time at this temperature caused degradation of the product. Of several structural analogues the *p*-methyl one was the internal standard of choice.

The amine was extracted from alkaline samples with dichloromethane and, after evaporation, reconstituted in methyl isobutyl ketone. From plasma the yields were lower than those from aqueous samples but the addition of hydroxylamine 30 min before the extraction process resulted in the same yields. Hydroxylamine probably acts as a competitor for carbonyl groups in the biological sample. In addition to the enhanced yields patients' samples extracted after hydroxylamine treatment were analysed with better precision.

With nitrogen-selective detection 500 nmol/l in a 0.5-ml sample could be quantified, which is well below the therapeutic levels. The method compared favourably with a liquid chromatographic assay.

INTRODUCTION

Tocainide [2-amino-N-(2,6-xylyl)propanoic acid amide], is an antiarrhythmic drug that is active after oral administration and has a suitable duration of effect. Previous bioanalytical data have been obtained with gas chromatographic methods using either flame-ionization[1] or electron-capture detection[2]. Liquid chromatography with fluorescence detection of a Dns derivative was reported to give high sensitivity[3], although methods without derivatization might suffice[4,5]. During clinical and biopharmaceutical studies of this drug large numbers of samples had to be assayed, which required a reliable and simple method that could be at least partially automated. This paper presents a gas chromatographic method based on nitrogen-selective detection of the Schiff base from tocainide and methyl isobutyl ketone. The derivatization reaction and experiences with the biological material are discussed.

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EXPERIMENTAL

Apparatus

A Hewlett-Packard 5730 gas chromatograph with a nitrogen-selective detector was used at 200°C with a 1 m × 2 mm I.D. glass column filled with 10% Carbowax 20M on 120–140-mesh Gas-Chrom Q. The temperatures of the detector and the injector were 300 and 200°C, respectively. The carrier gas (helium) and the detector gas (hydrogen–helium, 8:92) had flow-rates of 30 ml/min. Pre-purified air for the detector was used at 50 ml/min. A Hewlett-Packard Autoinjector was used.

Chemicals and reagents

Methylene chloride. Merck (Darmstadt, G.F.R.) p.a. (No. 6050) material was purified by distillation.

Methyl isobutyl ketone (MIBK). Merck p.a. (No. 6146) material was purified by distillation, then dried twice with and stored over molecular sieve 4A (Fluka, Buchs, Switzerland; 69834).

Tocainide standard solution. Tocainide (molecular weight 192.3) was obtained as the hydrochloride from the Department of Organic Chemistry, AB Hässle (Mölndal, Sweden). A 2.5-mg amount of tocainide hydrochloride was dissolved in and diluted to 100.0 ml with dilute hydrochloric acid (0.01 mol/l) to give a final concentration of 100 μmol/l. In this solution tocainide is stable for several months when kept in a refrigerator.

Internal standard. H 155/73 (molecular weight 206.3) was obtained as the hydrochloride from the Department of Organic Chemistry, AB Hässle. An aqueous solution was prepared in dilute hydrochloric acid (0.01 mol/l) to give a final concentration of 200 μmol/l.

Hydroxylamine solution. A 2.5-g amount of hydroxylammonium chloride (molecular weight 69.5) (Merck, p.a., No. 4619) was dissolved in 100 ml of water. This solution is stable for several months.

Glassware

All glassware was washed in a laboratory dish-washer at pH 12 (Extran AP12; Merck, No. 7563). The glassware was then rinsed at pH 2 (Extran AP21; Merck, No. 7559). The glass was finally rinsed with de-ionized water and dried at 60°C. All pipettes were silanized with dimethyldichlorosilane–toluene (1:9) overnight, washed with methanol and dried at 120°C.

Analytical procedure

The frozen sample is allowed to thaw at room temperature. After mixing, 0.5 g of plasma is transferred into a 15-ml centrifuge tube, 100 μl of the internal standard solution and 100 μl of the hydroxylamine solution are added and the sample is mixed. After standing for 30 min, 0.3 ml of sodium hydroxide solution (1 mol/l) and water to a total volume of 2.0 ml are added. This aqueous phase is extracted with 6.0 ml of dichloromethane. The tube is shaken mechanically for 10 min and centrifuged.

The organic phase is transferred into another 15-ml centrifuge tube and evaporated to dryness under a gentle stream of dry nitrogen. A 200-μl volume of MIBK is added to each tube, which is then tightly sealed with a screw-cap. The tube is placed in

a water-bath (85°C) for 10 min and then cooled with water to room temperature. A 3-μl volume of this solution is injected into the gas chromatograph.

Quantitative evaluation

A calibration graph was prepared by addition of various volumes (0, 50 and 200 μl) of the tocainide standard solution to 0.5 ml of plasma then analysing these samples according to the procedure given above. The peak-height ratios of tocainide to internal standard were calculated and the average of these values was then used for the quantitative evaluation of the samples taken for analysis.

RESULTS AND DISCUSSION

Tocainide is a primary amine (compound I in Fig. 5) that can be converted into derivatives that are more easily gas chromatographed than the free amine. Even if the primary amine itself can be chromatographed on stationary phases such as Carbowax 20M + potassium hydroxide, the handling of trace amounts of free amines can create adsorption problems in the steps before gas chromatography[6].

Primary and secondary amines can be acylated with perfluorinated reagents to give the corresponding amides, which are easily detected in picogram amounts using gas chromatography with electron-capture detection[7,8]. This approach has been reported for tocainide in small samples from rats[2]. Primary amines have also been derivatized in condensation reactions that are specific for this group of amines, *e.g.*, reaction with carbon disulphide and formation of Schiff bases with aldehydes and ketones[9]. The latter approach has mainly been used for qualitative purposes, the so-called peak shift technique[10]. Studies by Horning and co-workers[11–13] have shown the favourable gas chromatographic properties of the Schiff bases of several important amines. The separation of, *e.g.*, isomeric compounds can also be facilitated[13]. However, only a few quantitative methods have been reported. Amphetamine has been determined in dosage forms after condensation with cyclohexanone in a two-phase system[14]. Hoshika[15,16] studied the quantitative formation of Schiff bases from lower aliphatic primary amines with benzaldehyde and pentafluorobenzaldehyde.

The combination of a selective detection system such as the alkali flame-ionization detector with a simple derivatization reaction such as Schiff base formation could be used favourably for large series of samples that occur in pharmacokinetic studies.

Choice of condensing agent

Initial studies were performed with acetone as condensing agent. A single derivative was formed, which, however, co-eluted on the columns studied with a major peak originating from an endogenous plasma component. The choice then fell upon methyl isobutyl ketone, which is also available in good quality. Its volatility is lower than that of acetone, which is advantageous if the reaction mixture has to be stored for some time, such as in an auto-injector system.

Ketones were preferred to aliphatic aldehydes, which gave rapidly degrading products. As pointed out by VandenHeuvel *et al.*[13], an advantage of the stable ketones is their dual function as solvent and reagent. The reaction mixture can be injected directly into the gas chromatograph.

Reaction conditions

Schiff base formation is considered to be a two-step reaction (Fig. 1). This sequence has been suggested for the reaction between cyclohexanone and amphetamine based on infrared measurements[17].

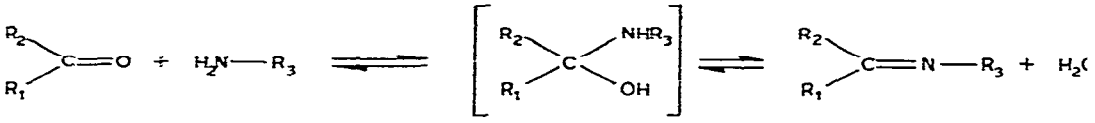

Fig. 1. Equation for the formation of a Schiff base.

Previous studies on Schiff bases (from ketones) for gas chromatography have demonstrated that the condensation reaction is a slow process[18]. With methyl isobutyl ketone the reaction at room temperature required at least 60 min for tocainide (Fig. 2A). Elevation of the reaction temperature increased the rate considerably (Fig. 2B and C). It was possible, however, even at 50°C to observe the start of degradation of the derivative formed. At 90°C the derivative was formed in apparently quantitative yield almost instantaneously (Fig. 2D), but after 120 min all of the derivative had disappeared. A number of extra peaks appeared early in the chromatograms from the

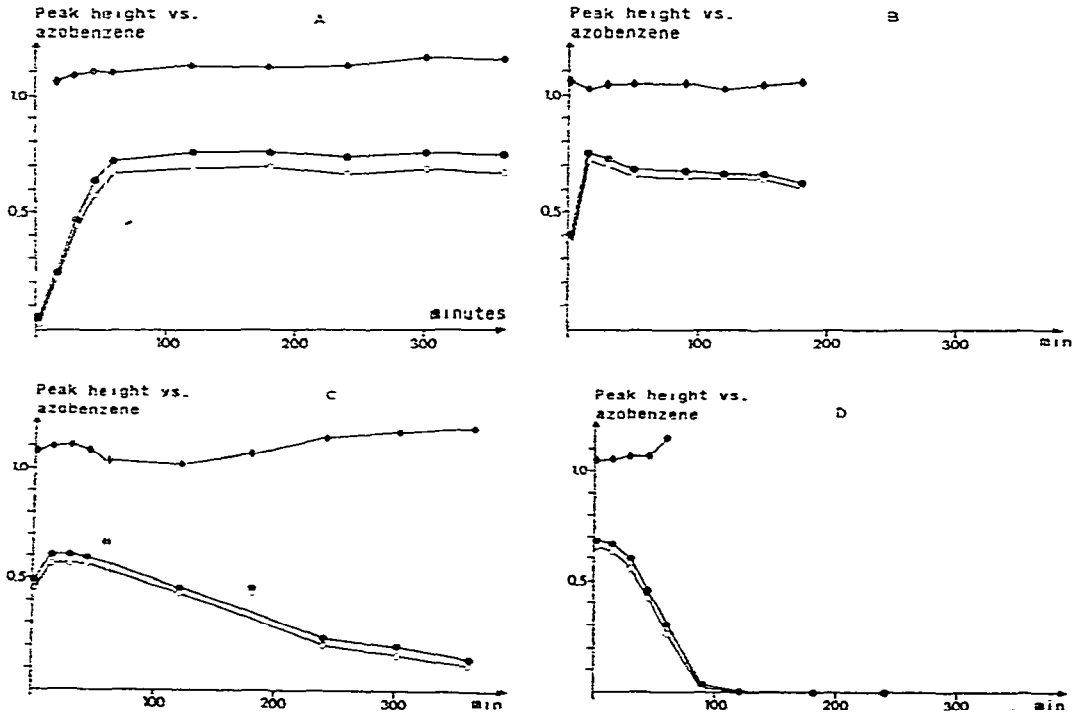

Fig. 2. Schiff base formation at (A) 22°C, (B) 50°C, (C) 70°C, (D) 90°C. ●, Tocainide Schiff base; ○, internal standard Schiff base; ◆, peak-height ratio.

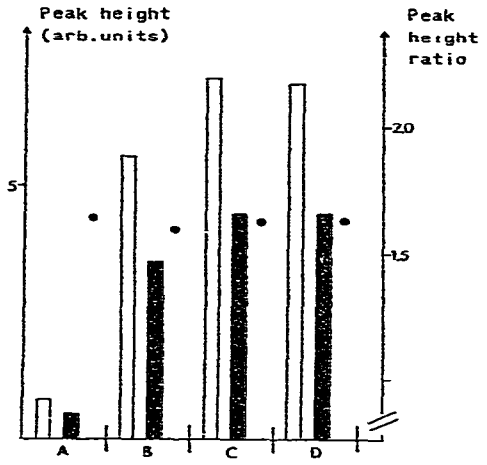

Fig. 3. Effect of pre-treatment of MIBK on peak height. (A) No treatment; (B) distillation; (C) distillation and treatment with molecular sieve; (D) as for C but with 1 % of water present. □, Tocainide Schiff base; ■, internal standard Schiff base; ●, peak-height ratio.

degraded solutions of the Schiff bases of tocainide and of the internal standard studied in parallel. Reaction at 85°C for 10 min was chosen for the method.

The formation of the derivative in the hot injector could be a possibility. This was ruled out, however, after an experiment with a so-called "sandwich" injection of methyl isobutyl ketone and tocainide dissolved in diethyl ether showed up only the free amine.

Quality of methyl isobutyl ketone

Initial studies with methyl isobutyl ketone gave high and reproducible yields of the Schiff base. It was observed, however, that single batches of the ketone gave fairly low yields, down to only 15% of the normal values. Purification of the ketone with drying agents such as a molecular sieve and/or distillation revealed that distillation was most effective in improving the yield (Fig. 3). The derivative is apparently sensitive to impurities in the ketone, as the derivatives rapidly disappeared from the ketone solution when a small volume of the distillation residue was added to it. The nature of these components has not been studied.

The presence of water would in principle effect the reaction, as seen from the reaction formula (Fig. 1). Hwang *et al.*[19] reported for the reaction of some fluorogenic aldehydes with primary amines that it was essential to remove the water formed during the reaction. On the other hand, Schiff base formation from cyclohexanone and amphetamine in a two-phase system was possible[14]. The present Schiff base seemed to be insensitive to the presence of water up to at least 1.5%, as the same yield was obtained as with the dried solvent (with less than 0.01 % of water (Fig. 3); see also the study of Bergel *et al.*[17] on amphetamine and acetone.

Stability of the derivative and reaction yield

As indicated above, the derivatives are prone to decomposition at elevated temperatures. At room temperature the stability was sufficient to allow storage in the

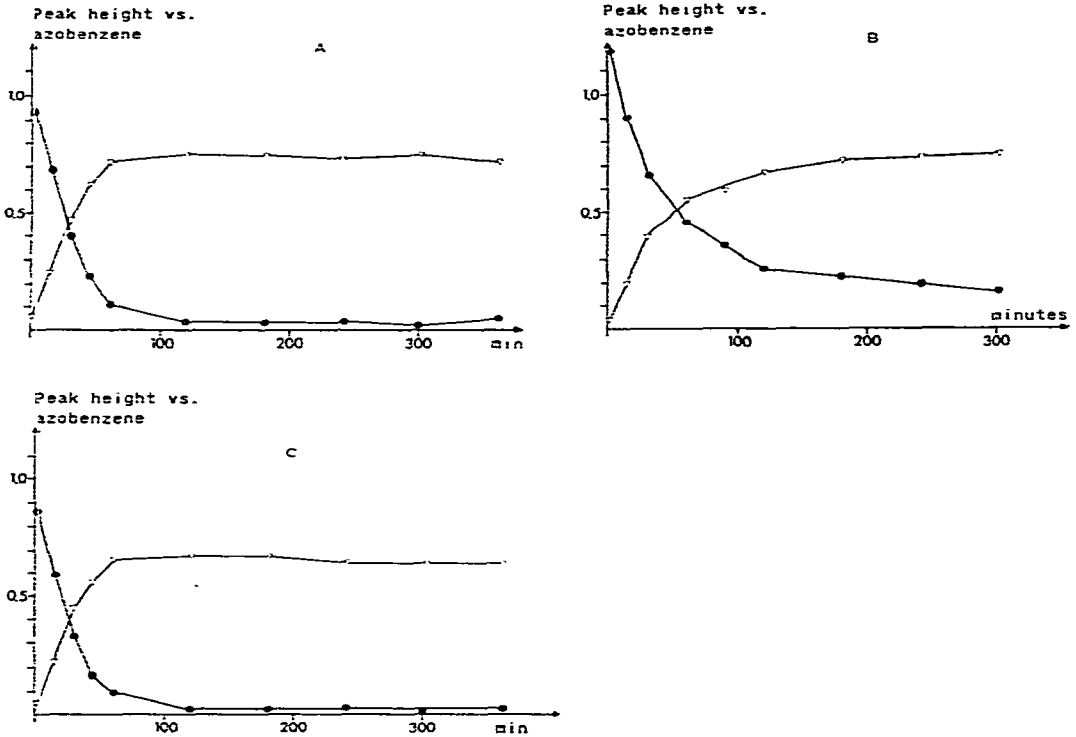

Fig. 4. Schiff base formation and disappearance of the primary amine at room temperature. (A) Tocainide (●) and its Schiff base (▽); (B) compound III (●) and its Schiff base (▽); (C) internal standard (II) (●) and its Schiff base (▽).

auto-injector cabinet for up to at least 24 h. Even if minor decomposition occurs this will not affect the quantitative evaluation as the internal standard used behaves similarly (see the peak-height ratios in Fig. 2B and C).

As soon as the ketone is removed from the reaction mixture or a dilution made with another solvent, reversal of the reaction begins[13]. The Schiff base from acetone and tocainide was available in crystalline form while the corresponding methyl isobutyl ketone derivative was an unstable oil. This makes it difficult to validate if the reaction is quantitative. A separate study (at room temperature) with some analogous compounds (see below) used chromatographic conditions more suitable for the free amine. This revealed that under the conditions employed the reaction yield is at least 95%, as less than 5% of the tocainide base was found (see Fig. 4A).

Reaction with structural analogues

Studies with four analogous compounds (Fig. 5) revealed some interesting differences (Fig. 4A–C). In these studies both the derivative and the intact primary amine were monitored. Only compound II reacted with the same profile as tocainide (I). Compound III, with an ethyl instead of a methyl group in the side-chain, reacted more slowly, whereas compound IV, lacking an alkyl substituent in that position, had a reactivity in between those of I and III. Compound V, with an additional methylene group attached to the amide carbonyl, was considerably less reactive than the other

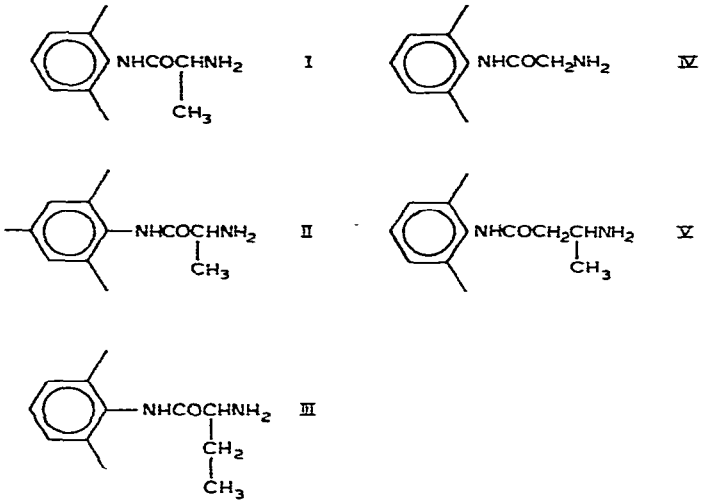

Fig. 5. Structural analogues. I, Tocainide (W 36095); II, internal standard H 155/73; III, W 36149; IV, W 49167; V, W 36196.

amines, despite its more basic nature (pK_a = 8.7) relative to tocainide (pK_a = 7.8)[19,20].

It is clear that an ideal internal standard should resemble tocainide in all respects around the functional group of interest for derivatization. Compound II, with an extra methyl group in the *p*-position, fulfilled this requirement and was the internal standard of choice. This will also be shown in other aspects below.

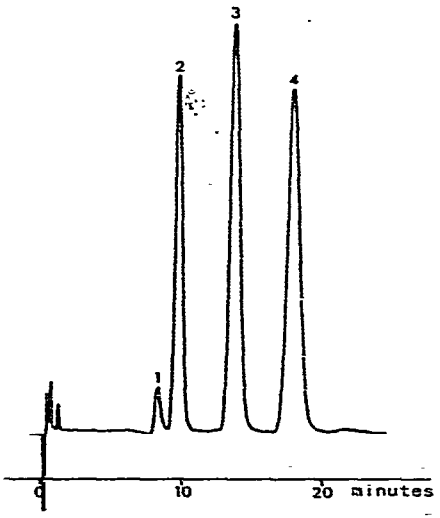

Fig. 6. Gas chromatogram from a derivatized extract of a patient's sample. 1 = Cotinine; 2 = tocainide Schiff base; 3 = internal standard Schiff base; 4 = caffeine.

TABLE I

RELATIVE RETENTIONS FOR THE COMPOUNDS STUDIED

For the structural formulas of the compounds, see Fig. 5.

Stationary phase	*Absolute retention of tocainide (min)*	*Relative retention**									
		Tocainide		*Internal standard*		*III*		*IV*		*V*	
		SB	*Base*	*SB*	*Base*	*SB*	*Base*	*SB*	*Base*	*SB*	*Base*
OV-17 (3%)	3.8	1.0	0.32	1.50	0.47	1.50	0.53	1.24	0.39	0.97	0.34
NP GSe (3%)	9.2	1.0	–**	1.29	–	2.16	–	1.40	–	1.22	–
OV-225 (3%)	5.4	1.0	0.57	1.42	0.76	1.70	0.98	1.39	0.72	0.96	0.65
Carbowax 20M (3%)	3.5	1.0	0.97	1.40	1.31	2.17	1.63	1.63	1.54	1.17	1.17
Carbowax 20M (10%)	18.9	1.0	0.92	1.42	1.29	2.03	1.57	1.52	1.43	1.10	1.14
Silar 5CP (3%)	2.6	1.0	0.77	1.42	1.04	1.88	1.46	1.58	1.12	1.00	0.92

* Relative retention *vs.* tocainide Schiff base (SB) with MIBK. All values refer to a column temperature of 190°C.

** The primary amine did not elute on this stationary phase.

TABLE II

EFFECT OF ADDITION OF HYDROXYLAMINE* TO PLASMA

Plasma sample (n = 5)	*Concentration of tocainide (μmol/l)*	*Peak height (arbitrary units)*						*Relative standard deviation of peak-height ratio (tocainide to internal standard) (%)*	
		Tocainide			*Internal standard*				
		Without hydroxylamine	*With hydroxylamine*	*Change (%)*	*Without hydroxylamine*	*With hydroxylamine*	*Change (%)*	*Without hydroxylamine*	*With hydroxylamine*
Spiked	3.4	3295	3605	+ 9	31715	35562	+12	3.1	2.3
	26.5	22954	24134	+ 5	31247	37568	+ 8	2.4	4.4
Patients' pooled	2.7	1679	1865	+11	33860	38790	+15	3.6	1.8
	25.3	20482	26413	+29	36431	43064	+18	5.3	1.4

* Final concentration of hydroxylamine in sample, 17.5 mmol/l.

Gas chromatography

The earlier documented excellent gas chromatographic performance of Schiff bases[13] was found for the derivatives in this study also (Fig. 6). Some stationary phases were studied with respect to their ability to separate tocainide, the internal standard and their Schiff bases. The results are given in Table I. Polar stationary phases are preferable to less polar ones such as OV-17, which shows some tailing of the base. Carbowax 20M (10%) was used in the method, as the other acceptable stationary phase, nitrogen-containing OV-225, used with the nitrogen-selective detector, showed much more noise. Silar 5CP did not show this behaviour. With the polyester stationary phase neopentylglycol succinate, the primary amine did not elute, probably owing to interaction with the carbonyl groups of the ester function.

The collector of the thermionic detector had a practical lifetime of about 1 month. At the end of this period the baseline began to drift and exhibited more noise.

The therapeutic levels of tocainide are within the range 10–30 μmol/l. It is possible to analyse this concentration without any difficulty with the present system as the limit of quantification is 0.5 μmol/l.

Extraction

Dichloromethane has been used as the extraction solvent in earlier studies with

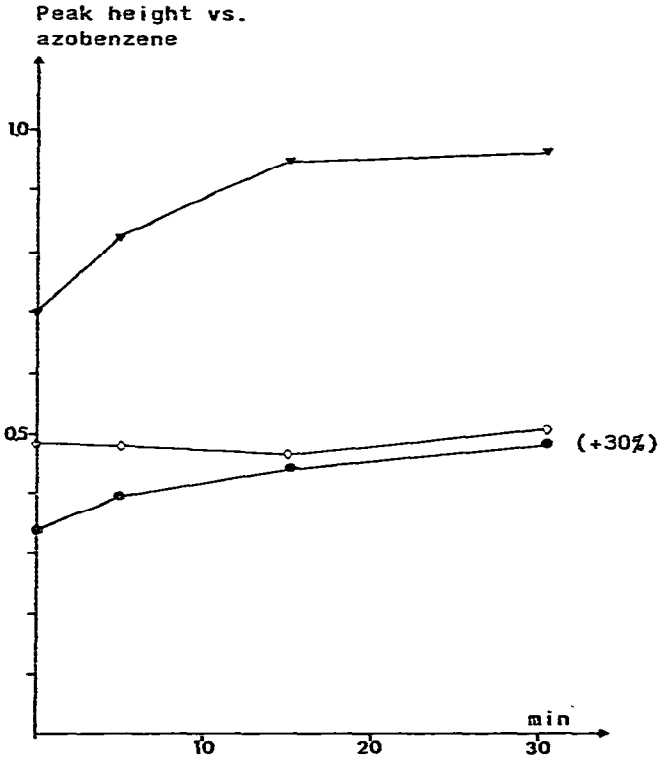

Fig. 7. Effect of time on the action of hydroxylamine. Conditions as under Experimental with 0.1 ml of hydroxylamine solution. ●, Tocainide Schiff base; ▼, internal standard Schiff base; ◇, peak-height ratio.

tocainide[2-4]. With a K_D value of 8 and a phase volume ratio (V_{org}/V_{aq}) of 3 the extraction yield is about 96%.

The quality of dichloromethane could sometimes affect the derivatization reaction in that a new compound appeared in the chromatogram. This was identified by gas chromatography–mass spectrometry as 3-(2,6-xylyl)-5-methylhydantoin. This cyclized compound, which is easily formed in a reaction with phosgene, has also been identified as a degradation product of a tocainide metabolite if the pH is raised above 12[21,22]. The presence of the extra peak was found only in dichloromethane that was not properly stabilized towards phosgene. The storage of the tocainide extracts is therefore not recommended. Smaller amounts of this by-product can be compensated for by the internal standard, which reacts in a similar way.

Applications to biological samples

The analysis of blank plasma samples spiked with tocainide differed from aqueous samples in that the recoveries were lower (80–95%). The reduction varied with samples of different origin. As the primary amine function might interact with endogenous compounds in the biological sample, the addition of a competing agent, hydroxylamine, was investigated. This resulted in recoveries that were comparable to those from pure aqueous solutions. The addition should preferably be made about 20 min before the sample is made alkaline and extracted (see Fig. 7). Again, the internal standard might compensate for minor differences between time zero and the alkalization (see the ratio of tocainide to internal standard in Fig. 7).

The results of a study of the effect of the addition of hydroxylamine to a spiked blank and to pooled patients' samples at two concentration levels are shown in Table

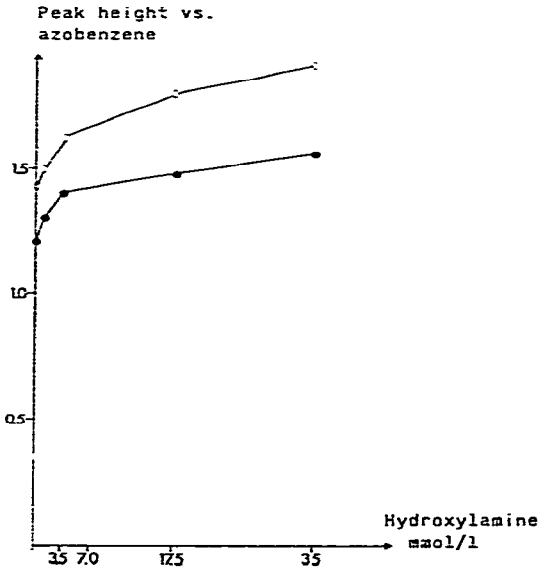

Fig. 8. Effect of final hydroxylamine concentration on the yield. Hydroxylamine acted for 30 min before extraction of the pooled patient sample. ◇, Tocainide Schiff base; ●, internal standard Schiff base.

II. The peak heights increase considerably by up to 30% for the high-level patients' samples. The amount of hydroxylamine needed was studied with a pooled patient plasma sample. From Fig. 8 it is evident that a final concentration up to 25 mmol/l increases the recoveries. In practice 17.5 mmol/l is used.

The addition of hydroxylamine also affected the repeatability of the results, especially for those from real patients' samples. Whereas aqueous and spiked samples analysed without any addition at the 25 μmol/l level had a relative standard deviation of 0.9 and 2.7%, respectively, the figure for a true biological sample at 10 μmol/l was as high as 14%. The relative standard deviation of the peak-height ratios obtained in the study shown in Table II demonstrates that the addition of hydroxylamine improves the precision for the real patients' plasma samples.

The observations on the effect of hydroxylamine imply that tocainide is present in the plasma samples not only in free form but also as a complex or a condensation product with some sample components. This resembles in a way what has been found for hydralazine, which reacts with pyruvic acid to give a hydrazone that can be co-determined under certain conditions[23,24].

With the present method the internal standard is added prior to the work-up procedure and is present during treatment with hydroxylamine. As can be seen in Fig. 7, even the absolute peak height of the internal standard increases during this treatment with the ratio between tocainide and the internal standard being almost constant. This indicates that the internal standard rapidly comes into equilibrium with the same components as tocainide and that the condensation products that they form are broken by the addition of hydroxylamine. It is not clear, however, why the patients' plasma samples differ from the spiked samples, as the latter are in fact equivalent to the way the internal standard is treated. Further studies are needed on this aspect.

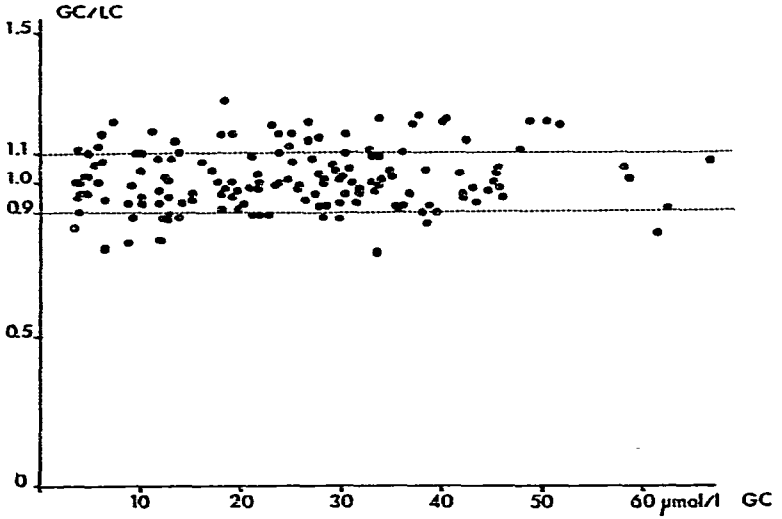

Fig. 9. Comparison of gas chromatographic (GC) and liquid chromatographic (LC) results. Mean value = 1.01; relative standard deviation = 10.1% (n = 160).

Validation of the method

The method has been used for the analysis of thousands of patients' samples with therapeutic concentrations in the range 10–30 μmol/l. The repeatability was evaluated for three levels, 50, 24 and 1.5 μmol/l, and was found to be 2.8, 3.7 and 10.7%, respectively ($n = 10$). The practical limit of quantitation for samples of 0.5 g is 500 nmol/l (corresponding to 0.1 μg/g) and one person can handle about 40 samples in a day.

The method has been compared with a liquid chromatographic assay[25]. The results agree fairly well within ±10% of the mean (see Fig. 9). The method has also been applied to urine samples.

With the use of this Schiff base no interferences were observed. However, as shown in Fig. 6, caffeine was often present in the samples, and sometimes also cotinine. The latter compound had a relative retention of 0.86 to tocainide.

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