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Note

Determination of moroxydine in biological fluids by electron-capture gas chromatography

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For the analysis of the biguanides buformin, metformin and phenformin, gas chromatographic methods have been used in the last few years [1—5]. They are all based on the cyclization of the biguanide by a suitable anhydride. High detection sensitivity has been achieved with electron capture [2] or with mass fragmentography [4]. Recently, an alkali-flame detector in the nitrogen mode was used and even that had the capability of detecting nanogram amounts of the drug [5].

Moroxydine has previously been analyzed by non-chromatographic methods, but recently a mass fragmentographic procedure was presented [6].

In this report a gas chromatographic method is described for the determination of moroxydine in biological fluids. The method is based on isolation by ion-pair extraction, formation of a triazine derivative by cyclization with chlorodifluoroacetic anhydride and gas chromatography with electron-capture detection. Emphasis is placed on the isolation of the rather hydrophilic moroxydine.

EXPERIMENTAL

Apparatus

A Varian 1400 gas chromatograph, equipped with a scandium ^{3}H electron-capture detector, was used with a 1.5 m × 1.8 mm glass column filled with 2% OV-225 on Chromosorb G. The column was conditioned at 300°C for 3 h and operated at 220°C with a nitrogen flow-rate of 30 ml/min. The detector and injector temperatures were 280°C and 230°C, respectively.

A Multi-Temp-Blok (Lab-Line Instruments) was used for the derivatization at 50°C.

Reagents

Trichloroacetic acid, hydrochloric acid 37% min., sodium dihydrogen phosphate, disodium hydrogen phosphate and toluene were all of analytical purity grade and obtained from E. Merck (Darmstadt, G.F.R.). Sodium hydroxide (analytical purity grade) was purchased from EKA (Bohus, Sweden). Tetrabutylammonium hydrogen sulphate (purum quality) was obtained from Fluka (Buchs, Switzerland). Methylene chloride (analytical purity grade) was obtained from Fisher (Fair Lawn, NJ, U.S.A.) and chlorodifluoroacetic anhydride from Bristol Organics (Hotwells Road, Bristol, Great Britain).

Bromothymol blue (BTB) indicator was obtained from Merck and purified by extraction at pH 8.5 with tetrabutylammonium hydrogen sulphate according to principles presented by Borg et al. [7].

Moroxydine hydrochloride was synthesized at KabiVitrum and found to be more than 99.0% pure as determined by titration and high-voltage paper electrophoresis. N-Aminoiminomethyl-1-piperidinecarboximidamide dihydrochloride was synthesized at KabiVitrum and used as internal standard.

[U-^{14}C]Moroxydine hydrochloride was obtained from the Radiochemical Centre, Amersham, Great Britain, and had a specific activity of 8.0 mCi/mmol (38.6 μCi/mg) and a stated purity of > 97%.

Method

Plasma and serum samples. A maximum 0.5 ml of the sample was mixed with 25—100 μl of internal standard solution containing 0.72 mg/l. The proteins were precipitated by mixing with 0.5 ml of 0.6 M trichloroacetic acid in 1 M hydrochloric acid. After centrifugation the supernatant was transferred to a centrifuge tube containing 1 ml of a BTB solution (8 × 10^{-3} M in phosphate buffer, pH 7.5, ionic strength 1). Sodium hydroxide (10 M) was added until the colour changed to green. The mixture was extracted with 8 ml of methylene chloride for 15 min and then centrifuged. The organic layer was filtered through a plug of silanized glass wool and evaporated to dryness on a boiling water bath. After cooling, 2 ml of methylene chloride and 20 μl of chlorodifluoroacetic anhydride were added and, after mixing, the solution was evaporated to dryness at 50°C. The residue was dissolved in 2 ml of toluene; 2 ml of 1 M sodium hydroxide were added and the mixture was extracted. After centrifugation 3—5 μl of the organic phase were injected into the gas chromatograph.

Urine samples. Urine samples were treated in the same way as plasma and

serum omitting the precipitation step. Samples containing low levels of moroxydine were purified by mixing with phosphate buffer (pH 7.5, ionic strength 1) and, prior to the addition of BTB, extracted with 8 ml of methylene chloride which was then discarded.

Preparation of standard curves. Standard curves were prepared by the addition of appropriate amounts of standard and internal standard to the same volume of blank serum, plasma or water (for urines) as the samples. The standard curves obtained by peak height measurements were linear between 0 and 1.5 mg/l moroxydine. From these standard curves the concentrations of the unknown samples were calculated.

RESULTS AND DISCUSSION

Derivatization

The structures of the s-triazine derivatives of moroxydine and internal standard with chlorodifluoroacetic anhydride, as shown in Fig. 1, were verified by gas chromatography—mass spectrometry. The preparation of the derivatives was easily performed during the evaporation procedure at 50°C. Between 1 and 100 μl of the anhydride gave a quantitative yield. Twenty microliters of the anhydride were used and found to be sufficient, even in the presence of co-extracted biological material.
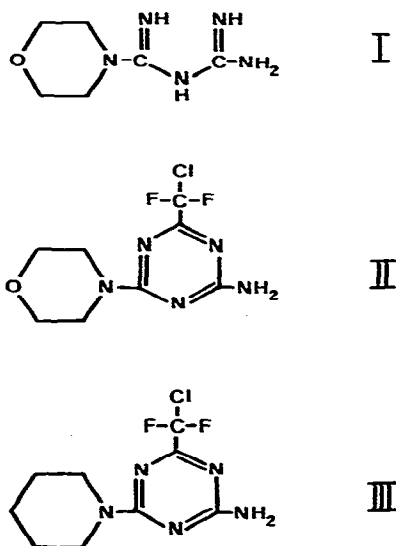

Fig. 1. Structure of moroxydine (I) and the derivatives of moroxydine (II) and of the internal standard (III).

Gas chromatography

The derivatives of moroxydine and the internal standard were run on several phases and it was found that non-polar phases like OV-1 gave tailing peaks. On OV-17, a moderately polar phase, the derivatives did not separate. The

best separation was achieved on a 2% OV-225 column, where the moroxydine derivative had a retention time of 5 min at 220°C. The relative retention of the internal standard derivative was 0.8. A gas chromatogram is shown in Fig. 2.

The derivative of moroxydine had an excellent electron-capture response. The minimum detectable concentration with a detector temperature of 280°C was 8×10^{-16} mole/sec, which means that about 17 pg of the injected derivative can be distinguished from the noise level.
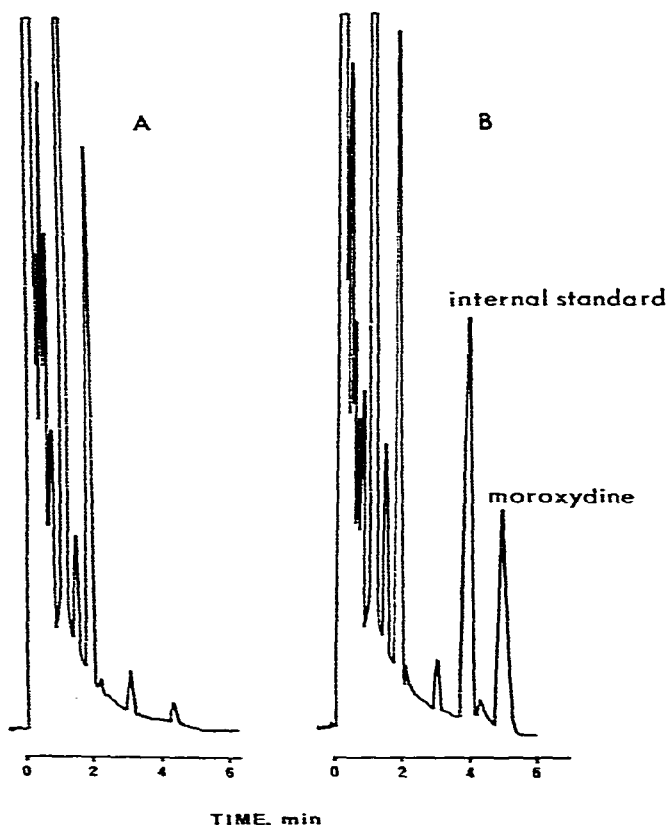

Fig. 2. Gas chromatograms from 100 μl serum. (A) Blank serum. (B) Serum containing 645 μg/l moroxydine. The injected amount of moroxydine derivative is 150 pg in 3 μl of solvent.

Extraction conditions

Garrett and co-workers [8, 9] studied the extraction conditions for three common biguanides as 1:1 ion pairs with BTB. The extraction of moroxydine as BTB ion pair was reported by Schill [10].

Initial experiments revealed that the quantitative extraction of moroxydine as base was not possible even when such highly polar solvents as 1-pentanol or 3-pentanol were used. The use of an aqueous phase with high ionic strength improved the extraction yields somewhat, but the extracts could not easily be derivatized. Furthermore, decomposition of moroxydine occurs in a strong-

y alkaline medium, which is why such extraction conditions should be avoided.

Schill [10] determined the extraction properties of the complex between noroxydine and BTB as the monovalent anion (HB⁻) and with methylene chloride as organic phase. The extraction constant was $10^{3.75}$ at pH 7.5, giving an extraction of 69% with a concentration of HB⁻ of $10^{-4}\,M$ (total concentration of BTB $= 3 \times 10^{-4}\,M$), the ratio between volume of organic phase and volume of aqueous phase being 4:1. Enhancement of the degree of extraction by increasing the total concentration of BTB is limited due to the formation of dimers and tetramers of the monovalent anion (HB⁻) [11].

According to the method, the BTB concentration is about $5 \times 10^{-3}\,M$ concentration of monovalent anion, HB⁻, is about $10^{-3}\,M$) and the phase volume ratio 5:1 in the extraction step. Calculations according to Schill [10], who used an ionic strength of 0.1, give a degree of extraction of 97%. However, the actual ionic strength was 1.0, which is why its influence on the extraction had to be elucidated. High blank values made photometric determinations impossible; thus radioactivity measurements using [U-¹⁴C]moroxydine were performed. An extraction of 80% was obtained with a moroxydine concentration of 16.5 mg/l.

The internal standard is more hydrophobic (due to the lack of the ether bond) than moroxydine and has an extraction constant of $10^{4.58}$. This corresponds to an extraction of 94% under the conditions given by Schill [10].

To increase the yield of moroxydine, a second extraction could be made. However, as samples analyzed with only one extraction gave the same results as those samples extracted twice, the more practical single-extraction procedure was preferred.

The absolute recovery including extraction and derivatization steps was about 70%, determined with radioactive moroxydine in a concentration of 58 μg/l.

Extraction from plasma and serum

Initial experiments showed that precipitation of the proteins with trichloroacetic acid in hydrochloric acid [2] gave higher recoveries than dilution with buffer. No interfering peaks originating from the serum were observed in the chromatogram. However, in some cases disturbances were noted which could be attributed to BTB.

Radioactivity measurements were used in some precipitation experiments, where it could be shown that sample volumes of 0.1 ml gave about 90% recovery of moroxydine in the supernatant, whereas 0.5 ml gave about 70% recovery.

As mentioned above, the absolute recovery from an aqueous solution was about 70%. When the precipitation losses are included the recovery from 0.5 ml of serum will be reduced to about 50%. However, these losses do not influence the final results as the internal standard compensates for some of the losses and the standard curves were prepared under identical conditions.

Extraction from urine

Urine with low levels of moroxydine contained some compounds that in-

terfered in the gas chromatogram. They could be removed if the urine sample at pH 7.5 was extracted with methylene chloride prior to the addition of BTB.

Recovery and precision

When known amounts of moroxydine were added to serum in a concentration of 505 μg/l and 0.15 ml was taken for analysis, the relative standard deviation was 2.9% ($n = 8$). Analysis of 0.5 ml of serum containing 21 μg/l gave a relative standard deviation of 9.3% ($n = 8$). The higher standard deviation reflects mainly the problems encountered in the precipitation of the proteins in the larger sample volume.

Applications to biological samples

The method has been applied to samples from biopharmaceutical and toxicological studies. An example of the time course curve from an oral dose of 800 mg of moroxydine hydrochloride is given in Fig. 3.
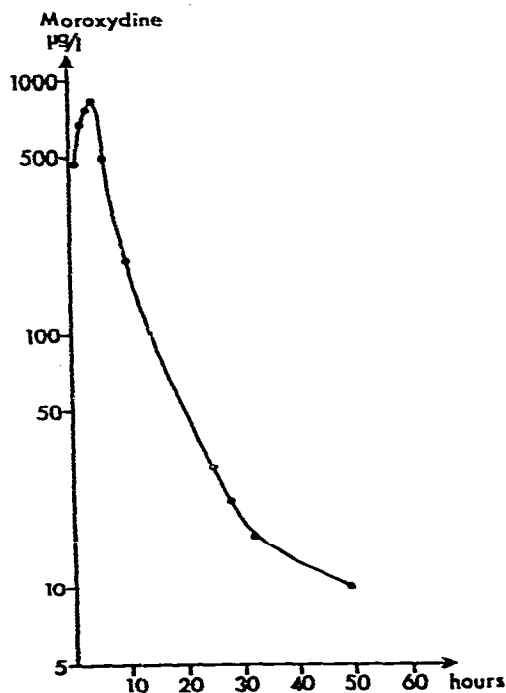

Fig. 3. Serum levels of moroxydine in a human volunteer after oral administration of 800 mg of moroxydine hydrochloride.

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