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DETERMINATION OF PIPERAZINE IN WORKING ATMOSPHERE AND IN HUMAN URINE USING DERIVATIZATION AND CAPILLARY GAS CHROMATOGRAPHY WITH NITROGEN- AND MASS-SELECTIVE DETECTION

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SUMMARY

A reliable routine method is presented for the determination of piperazine down to the sub-ppm level in aqueous solutions and in urine. The method includes a two-phase derivatization procedure with ethyl- or isobutyl chloroformate as the reagent, followed by a capillary gas chromatographic determination using nitrogen- or mass selective detection. The addition of ammonia ensured a quantitative recovery. Detection limits for piperazine in urine were *ca.* 20 ng/ml using nitrogen-selective and *ca.* 1 ng/ml with mass-selective detection. The calibration plots were linear in the investigated range, 100–10 000 ng/ml with nitrogen-selective and 30–3000 ng/ml with mass-selective detection. The precision was *ca.* 6% at a concentration of 300 ng/ml. Acid anhydrides were investigated as alternative reagents in the two-phase derivatization procedure, and heptafluorobutyric acid anhydride in aqueous solutions gave approximatively 100% recovery. However, in urine the recoveries of the investigated acid anhydride derivatives were unsatisfactory.

INTRODUCTION

Piperazine is a substance with many applications, *e.g.*, as a constituent of drugs, as an hardener in the manufacture of polymers and as an antioxidant. It and its derivatives are produced in large quantities, leading to considerable risks of work place contamination.

During the last 10–15 years, evidence of the potential health hazards of amines has been presented[1–4]. Hagmar *et al.*[5] have shown that piperazine in the air can induce asthma. Furthermore, some of the piperazine inhaled is transported with saliva to the stomach, where it partly reacts with nitrite forming the presumptively carcinogenic N-mononitrosopiperazine[6]. As a consequence, knowledge of the exposure levels in air and their relationship to human uptake and secretion is important. The large

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number of samples which need to be analysed in this case, both of the air and of body fluids such as urine, makes the development of simple routine methods highly desirable.

Older colorimetric methods as used by Rogers[7] and modified by Hanna and Tang[8] are not sufficiently specific and sensitive for analysis of piperazine at low concentrations in urine. A more recent method presented by Fletcher *et al.*[9] is based on acylation by acetic acid anhydride and subsequent analysis by gas chromatography (GC) on packed columns. However, the working range, 50–500 μg/ml of piperazine in urine, is much higher than that applicable for biological monitoring of occupational exposure. Furthermore, the use of acetic acid anhydride is questionable, since as suggested in their paper, acylated piperazine may also be formed by biotransformation.

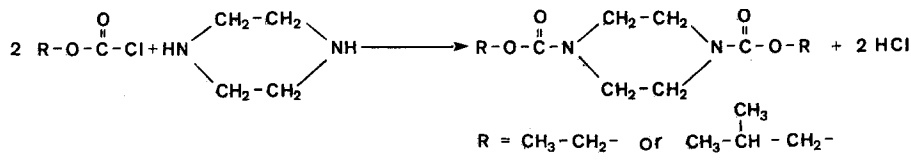
Methods used up to now in our laboratories for air samples (see ref. 5) do not have the desired simplicity for large numbers of samples. Furthermore, the precision of about 30% is not always sufficient. More recent methods for determination of free amines, including piperazine, in aqueous salt solutions developed at our laboratories[10,11] give a precision of about 2% and would seem applicable also to urine samples. However, with a matrix of urine there are certain drawbacks. Repeated injections result in changes of the chromatographic system, manifested as broadened peaks and prolonged retention times, necessitating frequent calibrations.

For routine measurements there is great value in a stable chromatographic system with no memory effects. Thus a work-up procedure such as extraction is in this case more or less necessary. If a derivatization step is also included, which usually gives a substance of lower basicity and polarity, it would be possible to use highly efficient capillary columns for the GC determinations. For piperazine, which is very soluble in water, a direct extraction to an organic solvent is not relevant. In such cases a two-phase derivatization which simultaneously combines extraction and derivatization is very attractive. Such procedures for amines in aqueous solutions have been developed with chloroformates[12] and acid anhydrides[13] and acid chlorides[14] as reagents.

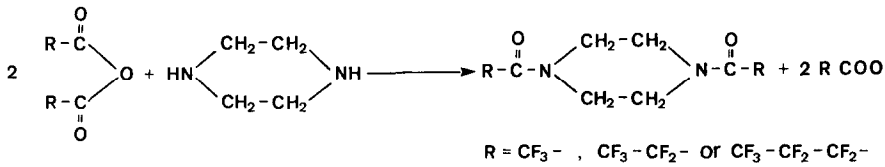
Air sampling of amines is generally performed in acidified aqueous solutions. For highly water-soluble amines such as piperazine this procedure gives 100% sampling efficiency. The determination of piperazine in both air and urine is accordingly performed from a water-based matrix. The possibility of using a similar work-up procedure for both air and urine samples has been taken into account in our work towards routine methods for piperazine determination.

In this paper we describe procedures for the routine determination of piperazine in aqueous solutions and in urine. They are based on two-phase derivatization followed by capillary GC using nitrogen-selective or mass-selective detectors. The derivatization reactions are illustrated below.

Carbamate formation:



Amide formation:



EXPERIMENTAL

Equipment

A Varian Model 3700 gas chromatograph was equipped with a Varian thermionic specific detector and a Grob-type on-column injection system with water-cooling designed and manufactured in our laboratories. Typical settings for the detector were: gas flow-rates, 6 ml/min of hydrogen and 200 ml/min of air; bead heating current, 5.3 scale divisions; bias voltage, 10 V; detector temperature, 270°C. Make-up gas for the detector (6 ml/min of nitrogen) was added to a carrier gas of helium. The carrier gas was dried over molecular sieve 5A and deoxygenated using an "Indicating Oxytrap" (Chrompack, Middelburg, The Netherlands). Chromatograms were recorded on Servogor Model 310 recorders and a Hewlett-Packard Model 3390 A integrator was used for peak evaluation. A Shimadzu GCMS-QP1000 EI/CI quadrupole gas chromatograph–mass spectrometer with a Shimadzu autosampler (AOC-9) was used for identification and quantitative routine analysis. The autosampler was used in connection with a Shimadzu splitless injection system (SPL-69). An Heidolph 2001 Rotavapor, rotating evaporator (Heidolph Elektro, Kelheim, F.R.G.) connected to an aspiration pump was used for evaporation. A Model 3E-1 centrifuge (Sigma, Harz, F.R.G.) was used for separation of phases.

Columns

A Duran 50 borosilicate glass capillary column (15 m × 0.32 mm I.D.) coated with PS-255 as stationary phase (film thickness 0.75 μm), produced in our laboratories[13], was used in the Varian GC system. Two other columns supplied by Chrompack were used both in the Varian and in the Shimadzu GC systems. These columns, Chrompack Aryl 17 CB (15 m × 0.32 mm I.D., film thickness 0.15 μm) and Chrompack CP-Sil 8CB (25 m × 0.32 mm I.D., film thickness 1.1 μm), are both chemically bonded.

Chemicals

Chemicals used were piperazine, piperazine hydrochloride, ethyl chloroformate, isobutyl chloroformate and toluene from Janssen (Beerse, Belgium), trifluoroacetic acid anhydride (TFAA), pentafluoropropionic acid anhydride (PFPA) and heptafluorobutyric acid anhydride (HFBA) from Pierce (Rockford, IL, U.S.A.) and di-*n*-butylamine from Fluka (Buchs, Switzerland).

Preparation of standard solutions of piperazine

Standard solutions were prepared by spiking alkaline aqueous solutions or urine with piperazine. These solutions were used for the investigations of recovery

and the linearity of peak height *versus* added amount of piperazine after performing the whole analysis procedure. Peak heights were compared to those of standards of the derivatives prepared as outlined below.

Synthesis of piperazine derivatives

Carbamate standards. Piperazine (10 g) was added to a mixture of 200 ml of 5 *M* sodium hydroxide and 200 ml of toluene, and about 5 ml of pyridine. The chloroformate reagent (40 ml) was added dropwise during 5 min with constant stirring. The toluene layer was separated and washed several times with distilled water. After evaporation of the toluene solvent in a rotating evaporator, the residue was dissolved in toluene–isooctane (1:1) and recrystallized at about −15°C.

Amide standards. Each amide standard was prepared by adding the acid anhydride reagent to a solution of piperazine in toluene. The completeness of the reaction was monitored by GC with flame ionization detection (FID). The solution was shaken with a 1 *M* phosphate buffer at pH 10 to remove the excess of reagent and acid formed. The toluene solution was separated and further diluted in toluene to the appropiate concentration of the amide derivative. Standard solutions prepared in this way are stable for at least 3 weeks at room temperature at concentrations corresponding to about 100 ng/ml of piperazine.

Two-phase derivatization and sample work-up procedure

With chloroformates as the derivatization reagents. A 1-ml aqueous sample was added to a mixture of 2 ml of 1 *M* phosphate buffer at pH 10, 2 ml toluene and 1 ml of 2.5% (w/w) aqueous ammonia. The mixture was shaken and 20 μl of chloroformate reagent were added. The mixture was shaken for 1 min and centrifuged at 4000 rpm for 40 min. A 1-ml aliquot of the toluene layer was evaporated for about 15 min. For the ethyl chloroformate derivative, the temperature was kept at 25°C and for the isobutyl chloroformate derivative, at 40°C. A 1-ml volume of toluene was added to the dry residue. The toluene solution contained an internal standard comprising the isobutyl chloroformate derivative of di-*n*-butylamine at the appropriate concentration. The toluene layer was analysed by GC. The same procedure was used for urine samples.

With acid anhydrides as the derivatization reagents. A 2-ml aqueous sample was added to a mixture of 2 ml toluene and 2 ml of 1 *M* phosphate buffer at pH 10. The mixture was shaken, 40 μl acid anhydride were added and the mixture was immediately shaken vigorously for 1 min. An aliquot of the toluene layer was analysed by GC.

RESULTS AND DISCUSSION

Standards

The identity of the piperazine derivatives was confirmed by gas chromatography–mass spectrometry (GC–MS). The purity of the derivatives was checked by GC using FID and was higher than 99%. For the chloroformate derivatives, the preferred products for routine analysis, the purity was further examined by elemental analysis. The experimental values for carbon, hydrogen and nitrogen differed by less than 0.2% from the calculated ones. The remaining amount of chloroformate reagent

was controlled by determining the chlorine content, which was found to be less than the detection limit or certainly less than 0.1%.

Derivatization reactions

Chloroformates as reagents. Parameters for the derivatization of different amines have been extensively studied in both single- and two-phase systems by Hartwig and co-workers[12,15,16]. In general, derivatizaton in a two-phase system with an appropriate pH in the aqueous phase will be faster than in a single-phase system. The choice of pH dependes on the basicity of the amine considered. For the reaction to occur at a reasonable rate, a considerable population of the nitrogen atoms must be unprotonated. For a volatile amine such as methylamine, the time required for quantitative reaction in a two-phase system at pH 10.3 was found to be between 20 and 280 min, depending on the reagent concentration[12]. For piperazine, with a pK_a value of 9.83 for the first step, we have found the reaction rate with isobutyl chloroformate in a two-phase system to be at least five times higher at pH 10.5 than at pH 7. At pH 10.5 the reaction is quantitative in less than 5 min. In a single-phase system with toluene as solvent the time for quantitative reaction of piperazine is in the order of 20–30 min. For very strong bases such as hexamethylenediamine (pK_a 11.86), no reaction occurs at pH 7 whereas the reaction is quantitative in about 10 min using a pH 12 phosphate buffer. The dependence of the reaction rate on pH may be used selectively to determine aromatic amines in the presence of aliphatic ones.

A basic compound soluble in an organic phase may be added to improve the neutralization of the hydrochloric acid formed and increase the reaction rate. We found such an addition to be necessary in order to get a quantitative recovery. Pyridine may be used but ammonia is even better, promoting 100% recovery for piperazine derivatives of both isobutyl chloroformate and ethyl chloroformate.

Acid anhydrides as reagents. Acid anhydrides have been used as derivatizing agents for amines in single-phase systems with good results[17–19]. Two-phase derivatization of some amines with PFPA has been demonstrated by Skarping *et al.*[13]. One example here is the reaction between 2,4- or 2,6-toluenediamine and PFPA, where quantitative recovery was obtained and the time required for complete reaction was less than 1 min. The derivatives were stable in toluene solution for several weeks. The usefulness of PFPA and some other perfluoro fatty acid anhydrides for the derivatization of piperazine is discussed below in the recovery experiments.

Choice of reagent. When determining piperazine in aqueous solutions, both chloroformates and acid anhydrides may be used. In samples, where disturbing peaks appear, a change from one type of reagent to the other may solve the separation problem. The work-up procedure using anhydrides is simpler, since it does not involve an evaporation step. Both types of derivatives exhibit excellent chromatographic behaviour.

In urine samples, only chloroformates can be used as reagents since acid anhydrides such as HFBA do not give quantitative recovery. This may to a large extent depend on the excessive foaming occurring when these reagents are shaken with urine. The loss using HFBA for the derivatization is in the order of 10–30% at piperazine concentrations of 1000 μg/ml and the precision of the analysis will accordingly be rather poor. The choice between the two chloroformate reagents is rather free; they both give fragment ions with high selectivity. The detection limits

are about equal with both nitrogen-selective and mass-selective detectors although they are somewhat better for the first eluting ethyl chloroformate derivative.

Work-up procedure

For chloroformate derivatives. The evaporation step to dryness described in the Experimental may serve several purposes, such as enrichment, removal of reagent excess or possibility to choose an optimum solvent for the subsequent GC or LC analysis. The most important of these, when using Varian's nitrogen-selective detector, is the removal of excess of chloroformate, since the injection of such a reagent influences the detector sensitivity. The response is at first rapidly increased, then decreases exponentially with time to a normal response in about half an hour. Whether other nitrogen-selective detectors give the same type of problem is not clear. Hartwig and co-workers[12] used an Hewlett-Packard thermionic nitrogen-sensitive detector but they did not mention this problem. The main reason for using the evaporation step also before mass-selective detection is the increase in capillary column lifetime. However, the possibility to enrich the component of interest may be important in future work on the analysis of N-mononitrosopiperazine at very low concentrations.

Other procedures for removal of the excess of reagent are available. Hartvig and co-workers[12] reacted the chloroformate with N,N-dimethylethylenediamine and extracted the derivative formed with an acidic phase. The addition of strong alkali to the aqueous phase, thereby catalysing the reaction of chloroformate to the corresponding alcohol, may be another alternative. Instead of removing the excess of reagent, one could use a standardized procedure for the chromatographic determinations with strict time control. We prefer the first alternative in order to avoid memory effects in the chromatographic system.

For acid anhydride derivatives. No additional work-up is needed after derivatization if the GC analysis is performed shortly thereafter. Otherwise the organic layer ought to be separated from the aqueous phase.

Mass spectra

Mass spectra of isobutyl- and ethyl chloroformate derivatives of piperazine were obtained for the purposes of identification and for chosing suitable fragment ions for quantitative analysis. Fig. 1 shows spectra of the derivatives obtained in the electron impact (EI) mode. Several characteristic fragments appear suitable for mass fragmentography, *e.g.*, for the ethyl chloroformate derivative the molecular ion (M) at *m/e* 230, the M − 15 fragment with loss of CH_3, the M − 29 fragment with loss of C_2H_5 and the M − 45 fragment with loss of C_2H_5O, and for the isobutyl chloroformate derivative the molecular ion (M) at *m/e* 286, the M − 15 fragment with loss of CH_3, the M − 73 fragment with loss of C_4H_9O and finally the M − 101 fragment with loss of $C_4H_9CO_2$.

Capillary GC with nitrogen-selective detection

Derivatization from aqueous solutions. After derivatization according to the procedures described, 1-ml portions of each organic solution containing the derivative were mixed together giving equimolar amounts of each component. A 2-μl volume of this solution was injected onto two different capillary columns, giving the

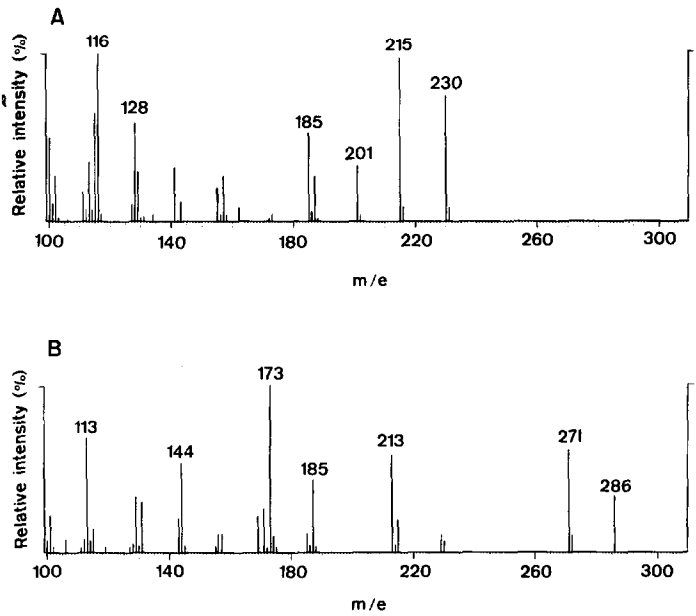


Fig. 1. Mass spectra of piperazine derivatives with ethyl chloroformate (A) and isobutyl chloroformate (B) obtained by electron impact ionization and positive ion monitoring.

chromatograms shown in Fig. 2. The chromatographic behaviour of all derivatives is excellent. Thus, in a situation where disturbing peaks may appear, the reliability of quantitative and qualitative analysis may be increased by using more than one derivative for the analysis or by changing from one column to the other.

Derivatization from urine. Fig. 3 shows chromatograms of isobutyl- and ethyl chloroformate derivatives of piperazine together with corresponding blank traces. Interfering peaks have been found, by analysing more than 50 different blank urine samples, to correspond to less than 20 pg/μl of piperazine, using either of these two reagents. The use of a more polar column such as Aryl 17 CB gave a somewhat better resolution of the ethyl chloroformate derivative of piperazine from possible interfering peaks. Accordingly, the accuracy and precision are somewhat improved and the detection limit somewhat lower.

Capillary GC with mass-selective detection

This technique has mainly been used for the two investigated chloroformate derivatives of piperazine. Fig. 4 shows some peaks of fragment ions of the derivatives at a piperazine concentration of 30 pg/μl with an injection volume of 1 μl. Several peaks are suitable for a quantitative analysis. In our routine analysis we have chosen the molecular ion (M) at *m/e* 286 for the analysis of the isobutyl chloroformate derivatives and that at *m/e* 230 for the ethyl chloroformate derivative.

Quantitative analysis

Chromatographic linearity. A linear variation of the peak height with injected

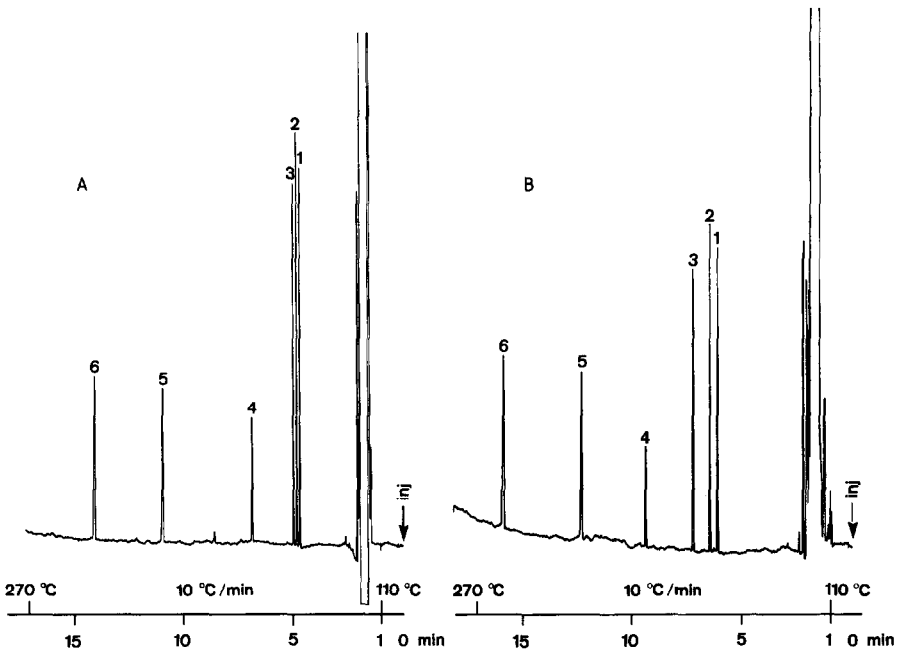


Fig. 2. Chromatograms of piperazine derivatives with nitrogen-selective detection: TFAA (1), PFPA (2), HFBA (3), ethyl chloroformate (5) and isobutyl chloroformate (6) at concentrations of 70 pg/μl with respect to piperazine. Internal standard: the isobutyl chloroformate derivative of di-*n*-butylamine (4) at a concentration of 130 pg/μl. Columns: (A) fused silica (15 m × 0.32 mm I.D.), bonded stationary phase Aryl 17 CB, film thickness 0.15 μm; (B) fused silica (25 m × 0.32 mm I.D.), bonded stationary phase CP-Sil 8CB, film thickness 1.1 μm. Injection: 2 μl on-column. Temperature programming as shown. Carrier gas (helium) at 0.7 kg/cm^2 (A) and 1.0 kg/cm^2 (B). Detector: bead heating current, 5.3 scale divisions; bias voltage, −10 V, temperature, 270°C. Flow-rates: hydrogen, 6 ml/min, air, 200 ml/min, make-up gas (nitrogen), 6 ml/min. Attenuation: $4 \cdot 10^{-12}$ A.f.s.

amount was found for all five piperazine derivatives both with nitrogen-selective detection and on-column injection and with mass-selective detection and splitless injection (see Fig. 5). The concentration range was 30–5000 pg/μl with respect to piperazine and 2 μl were injected using nitrogen-selective detection and 1 μl using mass selective detection.

Recovery. The recovery was studied at two concentrations, 300 and 3000 μg/l, by spiking aqueous solutions and urine with piperazine and performing the derivatization procedures as described in the Experimental. Peak heights were compared to those of standards by using nitrogen-selective detection.

For amide derivatives, which give unsatisfactory recovery and precision from urine samples, only aqueous solutions were considered. Of the reagents investigated (TFAA, PFPA, HFBA) only HFBA gives approximatively 100% conversion. This was confirmed by analysing the toluene layer containing the derivative by GC and comparing with standards. Corresponding figures were *ca.* 35% for TFAA and *ca.* 50% for PFPA. The recoveries could be increased to about 70% by two replicate additions of reagent. A change of the experimental conditions may further increase

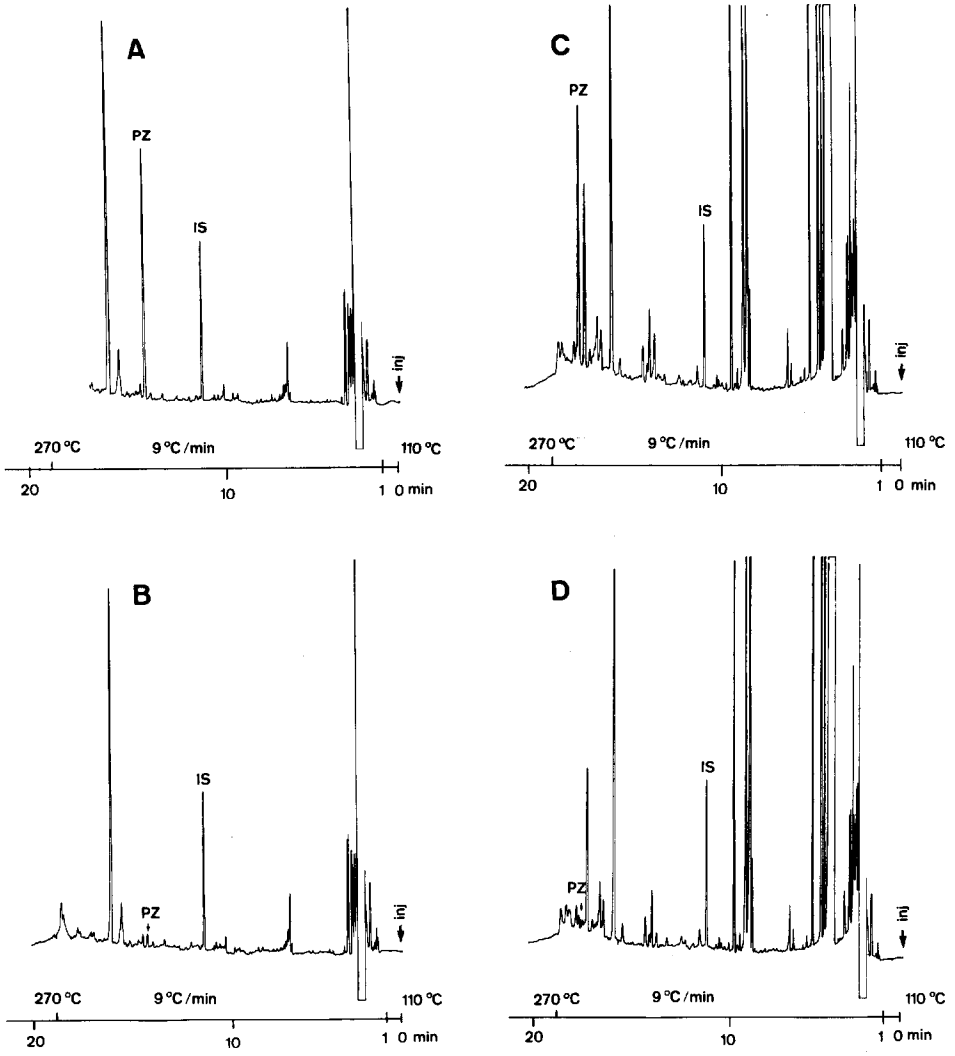


Fig. 3. Chromatograms of piperazine (PZ) at 500 pg/μl in urine treated with ethyl chloroformate (A) and isobutyl chloroformate (C) using nitrogen-selective detection. Internal standard (IS): the isobutyl chloroformate derivative of di-*n*-butylamine at concentrations of 200 pg/μl. Corresponding blank traces were for ethyl chloroformate in urine (B) and for isobutyl chloroformate in urine (D). Column: fused silica (25 m × 0.32 mm I.D.), bonded stationary phase CP-Sil 8CB, film thickness 1.1 μm. Carrier gas (helium) at 0.7 kg/cm². Temperature programming as shown. Attenuation: $8 \cdot 10^{-12}$ A.f.s. Other conditions as in Fig. 2.

the recovery. As quantitative recovery is obtained in toluene solution, the losses in this case may to a considerable extent depend on a competing hydrolysis reaction.

With ethyl- or isobutyl chloroformate as reagent, the recoveries were close to 100%, using ammonia as catalyst (0.4% of the total volume of the reaction mixture as described above). Figures obtained at 300 pg/μl were 101 ± 4% for ethyl chlo-

roformate and 98 ± 3% for isobutyl chloroformate at a 95% degree of confidence, based on twelve measurements. The amounts of ammonia and chloroformate reagents added are not critical. Variation of the concentration of ammonia between 0.33 and 1.65% and of the chloroformate reagents between 5 and 50 μl did not significantly change the recovery. A variation of pH between 8 and 10.5 in the phosphate-buffered urine or aqueous solution also did not change the recovery (with 0.33% ammonia and 20 μl chloroformate reagent).

Attempts were made to use pyridine, 0.33–1%, instead of ammonia as the catalyst. An 100% recovery was obtained for the ethyl chloroformate derivative, but in this case the urine blank exhibited a lot more peaks which may disturb the analysis. For the isobutyl chloroformate derivative only about 90% recovery was obtained. In this case the recovery was about the same as without any catalyst which was 91 ± 2% at a 95% degree of confidence based on fifteen measurements. For ethyl chloroformate without catalyst the recovery was only 75–80% with a relative standard deviation of 20–25% based on ten measurements. The lower recoveries without

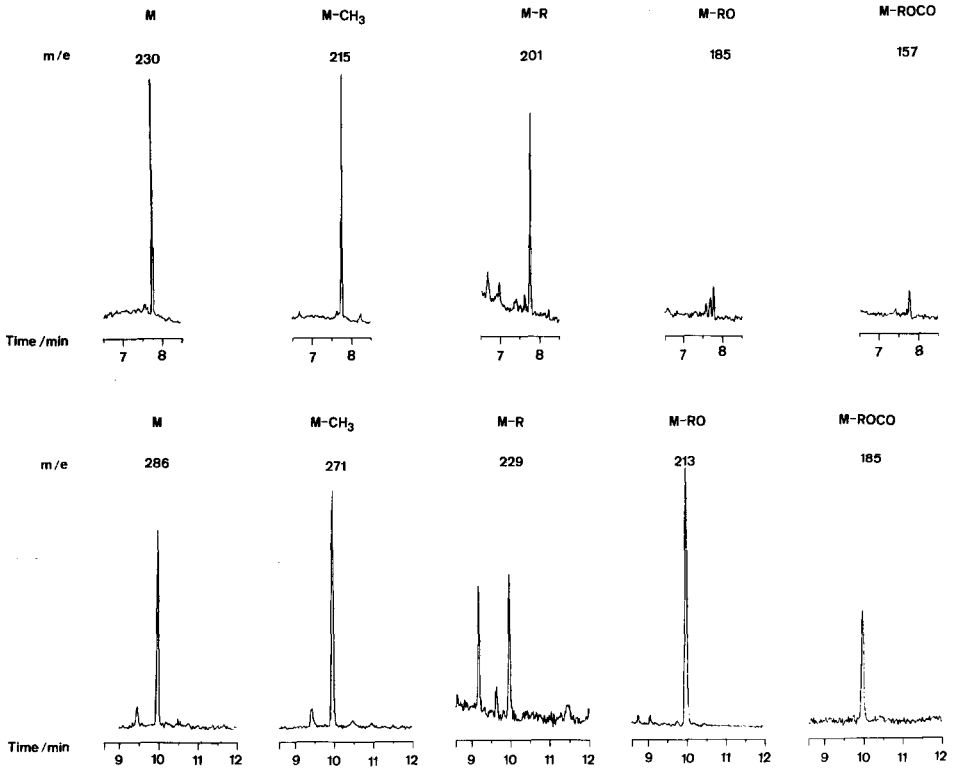


Fig. 4. Multiple ion detection of piperazine as chloroformate derivatives from urine corresponding to 30 pg/μl of piperazine. Topline: molecular ion (230) and fragments of ethyl chloroformate derivative. Bottomline: molecular ion (286) and fragments of isobutyl chloroformate derivative. Column: fused silica (15 m × 0.32 mm I.D.), bonded stationary phase Aryl 17 CB, film thickness 0.15 μm. Inlet pressure of helium: 0.1 kg/cm². Injection: 1 μl, splitless. Temperature programming: isothermal at 110°C (1 min) then raised at 10°C/min to a final temperature of 250°C. For the structure of the fragments, see the formulae given in text.

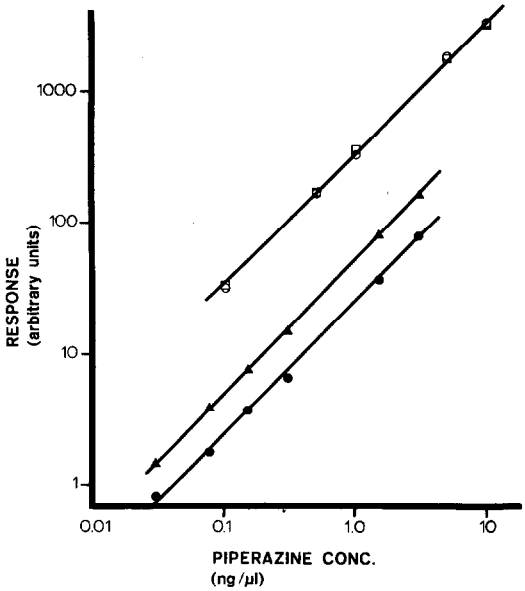


Fig. 5. Calibration curves for piperazine in urine as the corresponding chloroformate derivatives using nitrogen-selective or mass-selective detection: ethyl chloroformate derivative with nitrogen-selective (□) and with mass-selective detection (▲); isobutyl chloroformate derivative with nitrogen-selective (○) and with mass-selective detection (●). Peak height measurement was used with nitrogen-selective detection and peak area counts were used for mass fragmentography. The ratios between the values for the piperazine derivative and for the internal standard (the isobutyl chloroformate derivative of di-*n*-butylamine) are plotted. Chromatographic conditions as in Figs. 3 and 4.

catalyst are essentially due to losses during the derivatization step and not in the subsequent work-up procedures. This was confirmed by spiking the sample solution with piperazine derivatives of the two chloroformate reagents and performing the evaporation and work-up procedure. Calculated values of the recoveries were just 1–2% below 100%. These differences are of course not statistically significant, but nevertheless, small adsorption effects may well be present, leading to minor losses also during the evaporation step.

Calibration curves. Fig. 5 shows calibration curves in urine for piperazine derivatives of ethyl- and isobutyl chloroformate with nitrogen-selective or mass-selective detection. Different amounts of piperazine were added to aqueous solutions or to urine and the entire derivatization and work-up procedure was performed. For each piperazine concentration, three determinations were made with a duplicate injection into the gas chromatograph. Each point on the curve represents the average value. All plots are virtually linear in the concentration range investigated, *i.e.*, 100–10 000 pg/μl with respect to piperazine using nitrogen-selective detection and 30–3000 pg/μl using mass selective detection.

In aqueous solution in the corresponding concentration range, as expected, linear plots were obtained for all amide and chloroformate derivatives investigated, using both detectors. The chromatograms and the linear calibration plots show that any adsorption effect is of no practical importance.

Detection limit. Using nitrogen-selective detection the responses for all deriv-

atives are essentially the same. In aqueous solutions, where the chromatograms appear to be free from interfering peaks, the detection limits are less than 3 pg piperazine/μl. Values for HFBA presented in ref. 19 are about the same. In urine with the use of ethyl- or isobutyl chloroformate, small interfering peaks in the range of 0–15 pg/μl may appear, giving a detection limit in the order of 20 pg piperazine/μl.

Detection limits using mass-selective detection for the derivatives considered above were calculated as three times the noise level for peaks at a derivative concentration corresponding to 30 pg piperazine/μl urine (same concentrations as in Fig. 4). The molecular ion fragment peak of the ethyl chloroformate derivative gives a detection limit of 1.5 pg piperazine/μl and the corresponding value for the molecular ion of the isobutyl chloroformate derivative is 2 pg/μl. Some other fragment ions with good selectivity give about the same results. For the ethyl chloroformate derivative these are the ions at *m/e* 230, 215 and 201 where the most sensitive ion fragment at *m/e* 215 gives a detection limit of 1 pg/μl. Suitable ions for the isobutyl chloroformate derivative are those at *m/e* 286, 271, 213 and 185, where the most sensitive fragment ion at *m/e* 213 gives a detection limit of 1 pg/μl. Since no interfering peaks appear in this case, neither in aqueous solutions nor in urine, and the derivatives show no or very little adsorption in the chromatographic system, the detection limit depends only on the performance of the detector.

Accuracy and precision. When analysing a substance by GC after a work-up procedure and especially when this substance is initially present in a complicated matrix such as urine, the addition of an internal standard in the sample before the analysis is strongly recommended. Repeated injections may lead to changes in the separation process, *e.g.*, because of increasing adsorption on the column, or to changes in detector sensitivity with time. Changes in the injected amount with time, especially using splitless injection, may also occur. Such changes can usually be discovered and corrected for by using a combination of internal and external standards. The quotients of the peak heights and of the retention times can be used to evaluate the stability of the chromatographic system over a period of time. An increasing reliability in the identification thus results which improves the accuracy, especially

TABLE I

PRECISION IN THE GC ANALYSIS OF PIPERAZINE AS ITS ETHYL CHLOROFORMATE DERIVATIVE

Injected volume: 1 μl. Internal standard: the isobutyl chloroformate derivative of di-*n*-butylamine. An autoinjector was used for splitless injection and on-column injections were performed manually.

*Detection mode**	*Injection technique*	*Injected amount (ng)*	*Relative standard deviation (%)*		*No. of observations*
			Extern. std.	*Intern. std.*	
MF	Splitless	3	5.0	1.3	35
MF	Splitless	0.3	12.5	2.7	43
MF	Splitless	0.07	4.2	4.7	14
TSD	On-column	5	3.7	4.1	12
TSD	On-column	0.5	3.3	4.1	14
TSD	On-column	0.05	4.6	3.0	12

* MF = Mass fragmentography; measurement of the peak area of the molecular ion at *m/e* 230. TSD = Varian nitrogen-selective detector, with peak height measurement.

when the resolution between the substance peak of interest and peaks emanating from the matrix is not too good.

If the analyses are performed over a short period of time, during which the chromatographic system may be considered unchanged, one usually has to pay a price of lower precision when using an internal standard, compared to the use of only an external standard. In Table I, the precision has been calculated using an internal or an external standard. The value of an internal standard in cases where the chromatographic system changes with time is demonstrated. Considering the values at 0.3 ng/μl, with mass selective detection one finds that the use of an internal standard has greatly improved the precision (from 13 to 3%). As outlined above, the combination of an internal and an external standard may be used to ascertain reliable results even with small changes in the chromatographic system for a period of time. This implies relatively frequent injections of standards, but with the use of an autoinjector the sample throughput is still high. Normally we use a standard injection each fifth injection.

As internal standard we have used the isobutyl chloroformate derivative of di-*n*-butylamine and as external standard the analyte. The internal standard chosen behaves similarly to the piperazine derivatives in the chromatographic system and also with respect to the two detectors used for the analysis. A still better standard with respect to changes in the sensitivity of the mass selective detector would obviously be a deuterium-labelled piperazine derivative. However, the inadequate resolution of the unlabelled and labelled derivatives on the capillary column prevents its use with nitrogen-selective detection, which would necessitate different internal standards depending on the choice of detection mode.

The overall precision of the system, work-up procedure and GC analysis, is about 6% at a piperazine concentration of 300 pg/μl in urine, as shown in the recovery experiment. The contributions from the two operations are about equal as calculated from the results in Table I.

Choice of approach for the analysis

As shown above, several derivatives may be used for the analysis of piperazine in aqueous samples or in urine. The concentration of piperazine in aqueous samples after air sampling is usually high enough to permit a free choice between these derivatives. However, since the chloroformate reagents can also be used for urine samples, we have chosen chloroformates for routine analysis. Normally we prefer the ethyl chloroformate derivative due to its shorter elution time. The choice between detection modes, nitrogen-selective or mass-selective detection, is rather arbitrary. Somewhat higher precision is generally obtained using nitrogen-selective detection, but the detection limit is somewhat in favour of the mass-selective approach. However, the piperazine concentration in actual samples is usually so high that this difference in detection limit is of little or no importance. Splitless injection with an autosampler is preferred since it gives a higher throughput than manual on-column injection, about 45 samples per 24 h in the former case, compared to about 14 samples during an 8 h working day in the latter. The thermal stability of the piperazine derivatives formed is sufficient to permit splitless injection. This may not always be true, *e.g.*, chloroformate derivatives of N-mononitrosopiperazine show some degradation in the injection port, when using splitless but not with on-column injection.

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