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Gas chromatographic–mass spectrometric confirmation of selected benzophenones from benzodiazepines in human urine following automatic screening

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Abstract

This paper reports a simple, expeditious procedure for confirming the presence of benzodiazepines in previously screened positive urine samples. Samples are manually hydrolysed to the corresponding benzophenones in an acid medium. The hydrolysis products are continuously aspirated into a photometric flow system for screening; positive samples are then confirmed and benzodiazepines identified by using a different flow system that conditions the analytes for gas chromatographic separation and unequivocal mass spectrometric confirmation. Detection limits for benzodiazepines in the nanogram-per-millilitre region are thus achieved by using 0.5 ml of hydrolysed urine; also, repeatability, as R.S.D., is less than 6.5%. The high specificity and sensitivity of the proposed method enables the confirmation of different benzodiazepines in urine samples obtained from drug addicts and patients from a local hospital.

Keywords: Benzophenones; Benzodiazepines

1. Introduction

Benzodiazepines are the most widely prescribed sedative and hypnotic drugs in the world. Therapeutically, they were originally classified as ‘minor tranquillizers’ as, together with sedatives, they possess weak antianxiety actions [1,2]. The determination of benzodiazepines in biological fluids is of great interest as these compounds are involved in most intoxications.

Available screening methods for benzodiazepines are based on immunoassay, where an antibody reacts with a commonplace benzodiazepine metabolite present in the biological fluid [3–7]. They can detect a wide range of benzodiazepines and their metabolites in both plasma and urine samples. However, these screening procedures can rarely be used to quantify specific benzodiazepines unless a single benzodiazepine is present and its identity known. In this regard, the use of liquid (LC) [5,8] or gas (GC) [6,7,9–12] chromatographic techniques coupled to mass spectrometry (MS) allows the separation and unequivocal identification of the particular drugs present in a sample. These are currently the best choice for determining not only benzodiazepines, but also drugs in general, in biological samples. The equipment required is very expensive; however, its usage can be dramatically reduced by employing a screening method providing a yes/no response before confirmation. In this way, all samples are processed in the immunoassay screening method (which is convenient and fast), and only positive result need be confirmed by LC–MS [5] or GC–MS [6,7,12].

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Recently, our group developed a rapid, simple screening method for benzodiazepines in urine samples [13]; after hydrolysis of the drug, the urine sample is introduced into a continuous flow module for on-line derivatization and photometric detection of the hydrolysis products. However, every positive result from the screening system requires confirmation by using a procedure of a higher analytical rank. The aim of this work was to develop a GC–MS method for identifying and quantifying benzodiazepines yielding a positive result in the screening procedure. For this purpose, a continuous-flow system was designed that permits conditioning of the hydrolysed urine sample in order to remove the sample matrix and potential interferents as well as to change the aqueous medium into an organic one to make it compatible with the chromatographic system.

2. Experimental

2.1. Chemicals and standards

All reagents were of analytical grade or better. Methyl heptadecanoate [internal standard (I.S.)] was obtained from Aldrich (Madrid, Spain). Oxazepam, nitrazepam, diazepam, lorazepam, lormetazepam, flunitrazepam, prazepam, bromazepam, clorazepate, chlordiazepoxide and nonionic Amberlite XAD-2 were supplied by Sigma (Madrid, Spain). All other reagents (ethanol, chloroform, *n*-hexane, 1,4-dioxane, hydrochloric acid, sodium hydroxide and anhydrous sodium sulphate) were purchased from Merck (Darmstadt, Germany).

Stock standard solutions containing 1 mg/ml of individual analytes were prepared in Milli-Q purified water (clorazepate); 1,4-dioxane (oxazepam); chloroform (diazepam, flunitrazepam, nitrazepam and lormetazepam); ethanol (lorazepam, chlordiazepoxide and prazepam) or *n*-hexane (methyl heptadecanoate). All solutions were stored in amber glass bottles at 4°C. More dilute solutions were prepared as needed by diluting the stock standard solutions in 3 *M* HCl. The eluent (chloroform), containing 1 mg/l of methyl heptadecanoate (I.S.), was freshly prepared on a daily basis.

For the screening method, an aqueous 10^{-3} *M* $NaNO_2$ solution was prepared daily to diazotize benzophenones; the coupling reagent, 1-naphthol, was also made daily at a concentration of $6 \cdot 10^{-3}$ *M* in 6 *M* NaOH.

Blank urine samples were obtained from healthy individuals. Urine specimens collected from patients known to be receiving oral doses of benzodiazepines were screened and positive samples analysed by using the proposed confirmatory GC–MS method. All samples were handled as potentially infectious and frozen at −20°C immediately upon collection until analysis.

2.2. Components of the flow systems

The flow systems for the screening and confirmatory procedures (Fig. 1) were assembled from two Gilson Minipuls-2 peristaltic pumps (Worthington, OH, USA) fitted with poly(vinylchloride) and Solvaflex pumping tubes for aqueous and organic solutions, respectively. Three Rheodyne (Cotati, CA, USA) model 5041 injection valves, PTFE tubing of 0.5 mm I.D. for coils and standard connectors were also employed. The sorbent column for both flow systems was constructed by packing a commercially available column of 50 mm×2 mm I.D. (Omnifit, Cambridge, UK) with 50 mg of nonionic Amberlite XAD-2; small glass wool plugs were used on both ends to prevent material losses. A filter (for screening and GC–MS methods) was made from PTFE capillary (3 cm×4 mm I.D.) packed with cotton, which was fitted at the inlet of the aspiration sample channel to prevent the insertion of solid particles (produced by hydrolysis of the sample) into the flow system; the filter must be replaced after each working day. A Unicam 8625 UV–vis spectrophotometer (Unicam, Cambridge, UK) equipped with a Hellma flow-cell (path length 10 mm, inner volume 18 μl, Jamaica, NY, USA) was coupled on-line to the flow system for the screening procedure. An oil bath (Selecta, Barcelona, Spain) was also used.

2.3. Gas chromatographic–mass spectrometric conditions

Confirmatory assays were carried out on a Fisons gas chromatograph–mass spectrometer (GC8000/MD800, Thermo, Madrid, Spain) based on a quad-

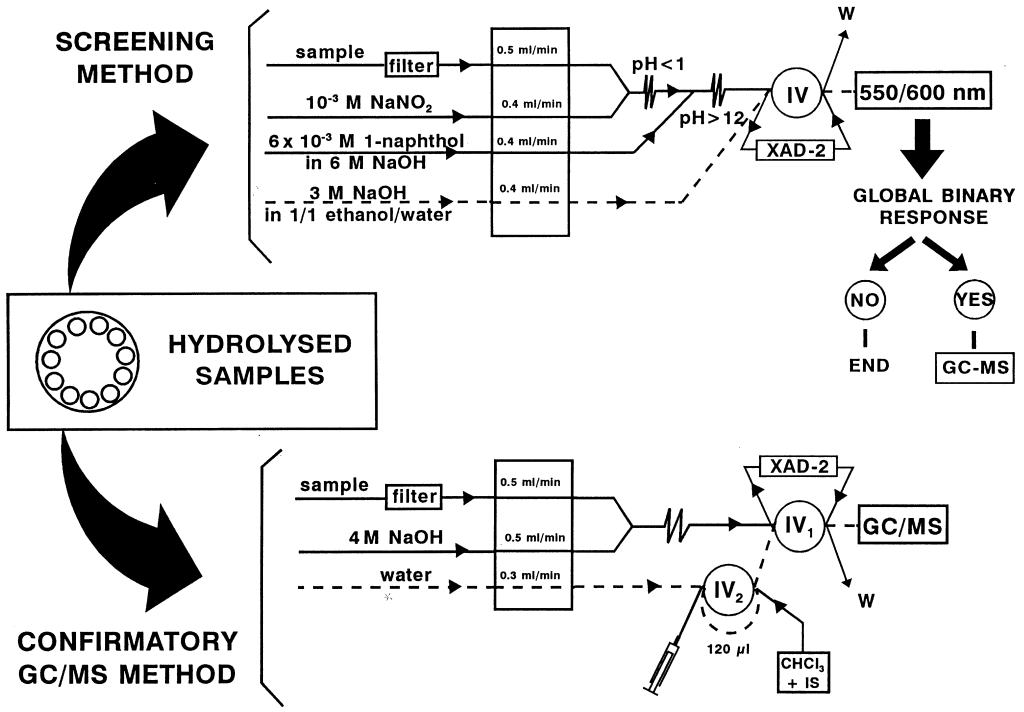

Fig. 1. Schematic diagram of the proposed set-up for the screening and confirmation of benzodiazepines in human urine. Screening method: IV, injection valve; reaction coils (25 cm×0.5 mm I.D); W, waste; GC–MS, gas chromatograph–mass spectrometer. Confirmatory GC–MS method: I.S., internal standard; conditioning coil (50 cm×0.5 mm I.D).

rupole analyser and a photomultiplier detector, and governed via MASSLAB software (also from Thermo). The flow-rate of carrier gas (helium, 6.0 grade, Air Liquide, Seville, Spain) through the column, a DB-5ms fused-silica capillary column (J&W Scientific, Cromlab, Barcelona, Spain) of 25 m×0.25 mm I.D. and 0.25 μm film thickness [stationary phase (5% phenyl)-methylpolysiloxane], was 0.9 ml/min. The injection port and transfer line temperature were maintained at 250°C. The ion source temperature was kept at 200°C. The mass spectrometer was operated in the scan mode (scan range 50–450). Electron ionization at 70 eV was selected as the ionization mode. Three characteristic ions for each analyte (only two for the I.S.) were monitored that included the base peak and the molecular ion for identification purposes. The *m*/*z* values selected for each benzophenone were as follows (quantitation mass underlined): oxazepam 77, 230, 232; diazepam 77, 245, 247; lorazepam 230, 265, 267; lormetazepam 244, 279, 281; prazepam 270, 285, 287; nitrazepam 195, 241, 242; flunitrazepam 211, 274, 275; methyl heptadecanoate 87, 284.

In order to optimize the GC separation of benzophenones, a mixture of ~10–20 mg/l of each benzodiazepine was hydrolysed in 3 *M* HCl and then extracted with chloroform. The organic phase, containing ~10 mg/l of each benzophenone, was spiked with the I.S. (methyl heptadecanoate), also at 10 mg/l. The temperature of the chromatographic oven was held at 120°C for 1 min, followed by ramping to 300°C at 10°C/min. The sample volume (1 μl) was injected in the split mode (1:25). Direct injection of bromazepam into the GC resulted in a tailing peak probably due to its thermal instability; also, the corresponding benzophenone was not detected.

2.4. Acid hydrolysis of benzodiazepines to benzophenones

The hydrolysis of the standard solutions of benzodiazepines and the urine samples was carried out as

described elsewhere [13], using 1 ml of aqueous standards or spiked/positive urine samples diluted with 1 ml of 6 *M* HCl in 10-ml stoppered tubes that were immersed in an oil bath at 100°C for 1 h. After cooling to room temperature, the urine samples were slightly turbid. Aliquots of 0.5–1.0 ml were continuously aspirated/filtered into the flow systems for screening or GC–MS. The hydrolysis efficiency ranged from ~50% (clorazepate and chlordiazepoxide) to ~99% (diazepam, prazepam, lormetazepam and oxazepam). The extent of hydrolysis of flunitrazepam and lorazepam could not be calculated because their corresponding benzophenones were not commercially available.

2.5. Photometric screening procedure

As can be seen in Fig. 1, hydrolysed urine samples (1 ml in 3 *M* HCl) were continuously fed into the flow system through the cotton filter. The filtered sample was merged with an aqueous 10^{-3} *M* $NaNO_2$ solution and the diazotized benzophenone was immediately formed (at pH<1) as a result. The stream was then mixed with the chromogenic reagent (viz, $6 \cdot 10^{-3}$ *M* 1-naphthol in 6 *M* NaOH) to obtain the coupled product (at pH>12). The dyes were retained on an Amberlite XAD-2 column and eluted (after rinsing the column with 1 ml of 3 *M* HCl) by using a solution of 3 *M* NaOH in ethanol–water (1:1, v/v). Eluted products were monitored at 550 or 600 nm. Between measurements, the sorbent column was conditioned with 1 ml of 3 *M* HCl aspirated through the sample channel. A volume of 1 ml of 3 *M* HCl was used as blank ($A \simeq 0.300$ units) and the peak height selected as the analytical measurement of the global binary response. Positive results must be confirmed by GC–MS as described in Section 2.6.

2.6. Confirmatory GC–MS procedure

The confirmatory GC–MS method is schematically depicted in Fig. 1. First, 0.5 ml of hydrolysed urine/standard solution containing benzophenones in 3 *M* HCl was continuously aspirated/filtered through the sample channel and mixed with a stream of 4 *M* NaOH, both at a flow-rate of 0.5 ml/min. A volume of 1 ml of 3 *M* HCl was employed as carrier to complete sample introduction (as well as to remove potential interferents and other organic compounds adsorbed on the column). The compounds were retained on an Amberlite XAD-2 column located in the loop of injection valve IV_1 and the matrix was sent to waste. Simultaneously, the loop of IV_2 (120 μl) was filled with eluent (chloroform containing 1 mg/l of I.S.) by aspirating the solvent with a syringe located at the end of the loop. By switching both injection valves, the loop contents, carried by a stream of water at a flow-rate of 0.3 ml/min, were passed through the column and the analytes eluted. The organic fraction was collected in a glass vial and 1 μl of the solution (previously dried with anhydrous sodium sulphate) was injected into the GC–MS system for analysis. The sorbent column was rinsed with 1 ml of ethanol, introduced via the water carrier channel and conditioned with 1 ml of 3 *M* HCl that was aspirated through the sample channel before the sample was inserted.

3. Results and discussion

Benzodiazepines are extensively metabolized in the human body, on absorption from the gastrointestinal tract, following one or more of three basic pathways, namely: N-desalkylation, C-hydroxylation and glucuronidation (see Fig. 2, top). Benzodiazepines alkylated on their 1-nitrogen are metabolized with loss of the alkyl group to form longer-acting, pharmacologically active compounds (e.g. nordiazepam or N-desalkylflurazepam [1,2].

3.1. Automatic screening system

The screening method is based on the diazotization of the benzophenones with nitrous acid and the subsequent formation of the coupled products with 1-naphthol (see Fig. 2, bottom). The proposed automatic screening system was optimized elsewhere [13]; the most significant variables are listed in Table 1. A solid-phase extraction step was implemented by including a sorbent column in the system in order to make the sensitivity of the method adequate for screening benzodiazepines in urine samples. For this purpose, conventional sorbents (viz. activated carbon, RP-C_{18} and nonionic Amberlite XAD-2) were tested [13]. Retention and elution were incomplete

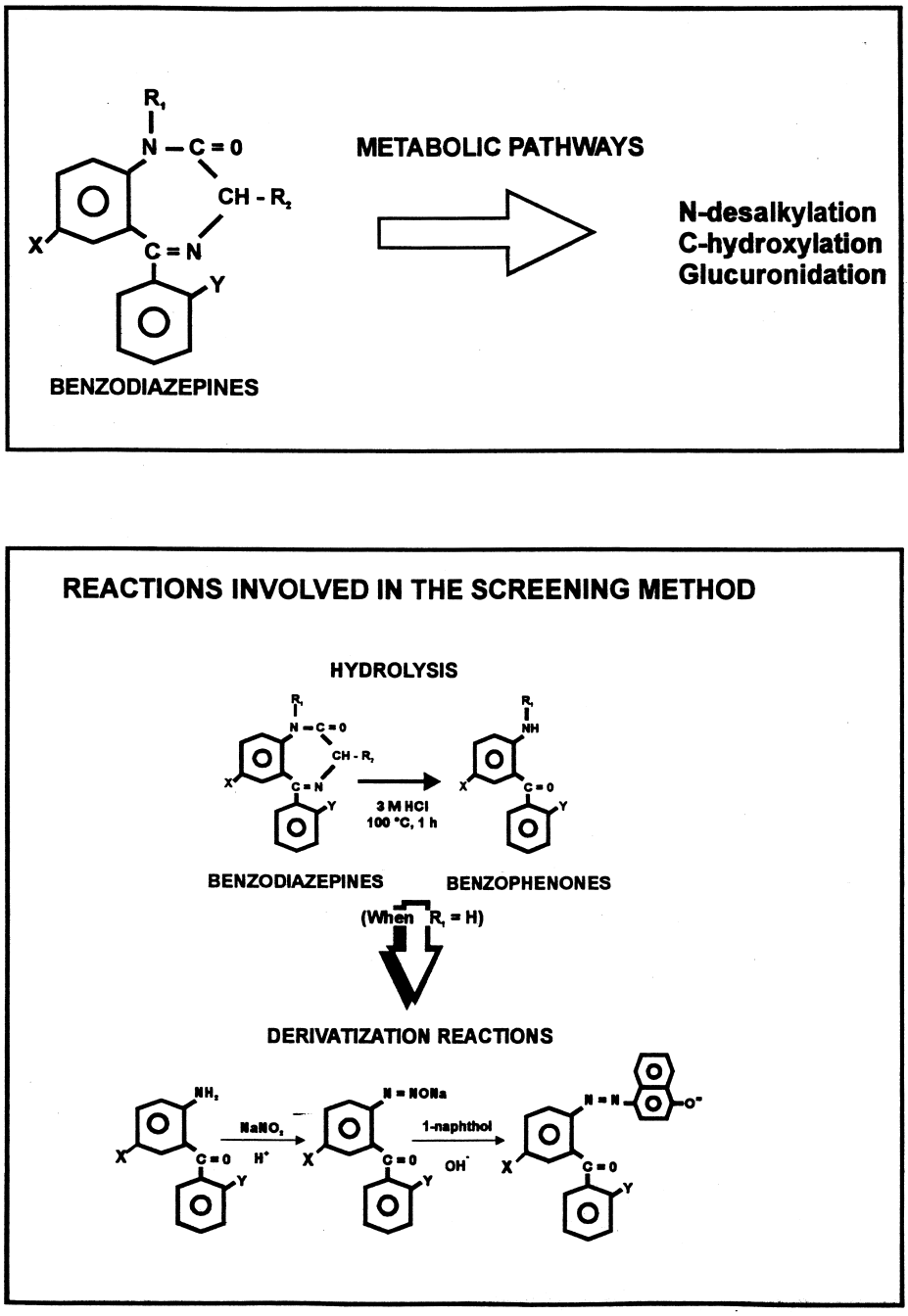

Fig. 2. Metabolic pathway of benzodiazepines (top) and reactions involved in the proposed screening method (bottom).

with RP-C_{18} and activated carbon, respectively. This problem is common using hydrophobic mechanisms of adsorption (viz. RP-C_{18} and activated carbon) on hydrolysed biological samples; therefore, better results were obtained with Amberlite XAD-2. Finally, an alkaline medium (NaOH) was required to im-

Table 1
Chemical and flow variables of the screening and GC–MS methods for benzodiazepines

Variable	Optimum range	Selected value
Screening method		
Hydrolysed sample pH, *M* HCl	2–4	3
$NaNO_2$ (*M*)	$5\cdot10^{-4}$–$5\cdot10^{-3}$	10^{-3}
NaOH (*M*)	4–8	6
1-Naphthol (*M*)	$2\cdot10^{-3}$–10^{-2}	$6\cdot10^{-3}$
pH of eluent (ethanol–water, 1:1, v/v), *M* NaOH	2–4	3
Sample flow-rate (ml/min)	0.4–0.6	0.5
$NaNO_2$ flow-rate (ml/min)	0.3–0.4	0.4
1-Naphthol flow-rate (ml/min)	0.3–0.4	0.4
Reaction coils length (cm)	10–200	25
Amount of Amberlite XAD-2 (mg)	40–100	50
GC–MS method		
NaOH (*M*)	3–6	4
Amount of Amberlite XAD-2 (mg)	40–100	50
Conditioning coil length (cm)	25–200	50
Eluent volume (μl)	110–120	120
Sample flow-rate (ml/min)	0.3–0.6	0.5
NaOH flow-rate (ml/min)	0.3–0.6	0.5
Eluent flow-rate (ml/min)	0.1–0.4	0.3

prove elution of retained dyes which was prepared in ethanol–water (1:1, v/v). Despite the simplicity and reliability of the proposed screening method, demonstrated in simple chemometric terms, it possess several problems, namely: differences in hydrolysis efficiency among benzodiazepines (50–99%) preclude the use of a single calibration graph (in molar concentrations) for all; also the fact that it responds to all benzodiazepines restricts its quantitation use to those cases where a single benzodiazepine is present and its identity known. Exceptionally, this method is specific to nitrazepam because its coupled product is blue (λ=600 nm) while the others are all pink (λ=550 nm).

3.2. Gas chromatographic–mass spectrometric confirmation

The above-described screening method requires confirmation of positive results owing to its non-specificity and inability to identify or quantify benzodiazepines. According to the literature [12,14], benzophenones can be employed to confirm positive samples. This results in several advantages such as an increased thermal stability of benzophenones relative to the parent benzodiazepines and the higher sensitivity obtained by using a hydrolysed urine sample. Therefore, the proposed confirmatory procedure uses a simple flow system based on liquid–solid extraction as sample pretreatment and GC–MS for the separation and identification of the benzophenones (and the original benzodiazepines) present in the urine sample. Initially, a flow system similar to that depicted in Fig. 1 and hydrolysed samples were used to optimize experimental chemical and flow variables potentially influencing drug recoveries.

First, the optimum conditions for retention on Amberlite XAD-2 were studied. Each benzodiazepine was spiked to a urine blank at a concentration of 2–4 μ*M* (oxazepam, diazepam, lorazepam, nitrazepam, lormetazepam, prazepam and flunitrazepam) which was hydrolysed. After hydrolysis, the sample was in a 3 *M* HCl medium whereas retention on the sorbent was favoured by an alkaline pH. A stream of 4 *M* NaOH was thus used at the same sample flow-rate to provide a pH above 12 after the mixing point prior to retention. Several sorbents were assayed in order to encompass a wide range of conditions. As in the screening method, Amberlite XAD-2 gave the best results; RP-C_{18} produced incomplete retention (~50%) and activated carbon irreversible retention. The amount of sorbent was varied between 40 and 100 mg (larger amounts

increased the pressure inside the flow system). This variable had no effect on benzophenone adsorption, so, the same column as in the screening method was adopted. Several organic solvents of variable polarity were assayed as eluents for retained benzophenones, namely: methanol, ethanol, *n*-hexane and chloroform. Ethanol, methanol and chloroform exhibited the best eluting properties. Chloroform was selected on account of its increased selectivity. The retention pH was studied from values of 2 to 12. All benzophenones were efficiently retained (~98% efficiency) when the sample pH was higher than 7. In contrast, benzodiazepines have no common retention pH values where the efficiency of the retention was maximal for all of them. In this context, the use of benzophenones is also of advantage.

The flow variables studied were the length of the sample conditioning coil; the sample, alkaline solution and eluent flow-rates; and the volume of eluent (chloroform). The length of the sample conditioning coil was minimally influential on the chromatographic signal as the neutralization reaction was very fast. A coil of 50 cm was used to ensure homogenization of the sample before it reached the sorbent column. The flow-rate of the sample (0.5 ml) and the 4 *M* NaOH solution were varied simultaneously in order to maintain a constant final pH and ionic strength. Changes in these flow-rates between 0.3 and 0.6 ml/min resulted in very small variations in the signal; at higher flow-rates, the adsorption efficiency was decreased through the decreased residence time. The selected value for each variable is given in Table 1. A water stream was used as carrier for the eluent (chloroform), for compatibility with the pumping tubes and immiscibility between the two phases. Benzophenones were eluted, with no carry-over at volumes above 110 μl. The water flow-rate resulted in very small variations in the elution efficiency up to 0.4 ml/min; at higher flow-rates, the efficiency decreased through decreasing residence time of chloroform in the sorbent column. Under the selected conditions, each sample remained inside the pretreatment flow system for about 4 min.

3.3. Linearity, sensitivity and precision of the GC–MS method

Analytical curves for hydrolysed standards of benzodiazepines at different concentrations (0.02–7 μ*M*) prepared as described in Section 2.4 were obtained by plotting the analyte-to-I.S. peak area ratio against the analyte concentration. Methyl heptadecanoate was used as I.S. in preference to others (bromhexine, amylocaine or papaverine) because it was found to be compatible with the flow system and the chromatographic behaviour of the analytes; it was added to the eluent at a concentration of 1 mg/l. The results obtained are listed in Table 2. The calibration graph for oxazepam was used to quantify this benzophenone in all the urine samples where it was found, whatever its origin [as a metabolite for another benzodiazepine (ketazolam, clorazepate, chlordiazepoxide), as the benzophenone obtained by hydrolysis of some benzodiazepines (clorazepate, chlordiazepoxide) or directly from the metabolic pathway (alprozolam)]. Detection limits, calculated as the minimum concentrations providing a chromatographic signal three times higher than background noise are also given in Table 2; they ranged from 1 (oxazepam and diazepam) to 70 ng/ml

Table 2
Figures of merit of the calibration graphs for the identification/quantitation of benzodiazepines by GC–MS

Compound	*m/z* Quantitation value	Slope	Intercept	Linear range (μ*M*)	Detection limit (μ*M*)	R.S.D. (%)
Oxazepam[a]	230	0.193	0.034	0.02–4.3	0.004	4.5
Diazepam	245	0.371	0.022	0.02–4.1	0.004	4.5
Lorazepam	265	0.126	0.034	0.15–6.2	0.062	6.5
Lormetazepam	244	0.254	−0.019	0.20–6.0	0.045	5.1
Prazepam	270	0.346	−0.034	0.15–4.9	0.054	6.3
Nitrazepam	241	0.124	0.018	0.21–6.2	0.062	5.7
Flunitrazepam	274	0.113	0.015	0.35–7.4	0.120	6.3

[a] Ketazolam, clorazepate, chlordiazepoxide and alprazolam (via benzophenone obtained after metabolism) were all quantified as oxazepam.

(flunitrazepam). The precision of the method, expressed as relative standard deviation (R.S.D.), was checked on 11 individual samples containing 2 μM concentration of the analytes and found to range from 4.5 to 6.5% (intra-day) and from 6.0 to 8.5% (inter-day). The sensitivity of the method was satisfactory for determining the drugs at the nanogram-per-millilitre level in urine; also, the precision was quite high in all instances.

3.4. Application to human urine

A recovery test was performed with spiked human urine to validate the GC–MS method. It was done at three different concentrations (1, 2 and 4 μM for all benzodiazepines except oxazepam and diazepam, which were tested at 0.5, 1 and 2 μM). High recoveries were obtained in all instances; mean values ranged from 91.2 to 102.5%. No interference from the matrix was observed at the m/z values used for identification and quantitation.

The proposed GC–MS method was used to confirm and identify the presence of benzodiazepines in previously screened positive urine samples. Samples were collected from individuals engaged in a detoxification program and from hospital patients. Each was supplied with the pharmacologic profile of the patient. After the screening method (~20 samples), only those containing benzodiazepines gave positive responses (9 samples); the results were confirmed by GC–MS and are listed in Table 3. Samples were suitably diluted. The proposed confirmatory method allows the individual identification and, in most cases, the quantitation, of benzodiazepines. The benzophenone of oxazepam was present in most of the samples (no. 4 excepted) as it is the final metabolic product of the parent benzodiazepine (clorazepate, chlordiazepoxide, ketazolam) or the benzophenone produced by the metabolic pathway has the same structure (alprazolam). The hydrolysis product for some standards of benzodiazepines (flurazepam, flunitrazepam, lormetazepam, diazepam and prazepam), could not give a positive result in the screening test because it contained no free amino group for derivatization. However, patients' samples behaved differently because the final metabolite was the N-desalkylbenzodiazepine, which contains the required primary amino group [2,15]. In this case, quantitation by the GC–MS method is impossible unless the urine contains some of the parent drug initially administered because the compounds obtained upon hydrolysis of standards and employed to construct the calibration graphs are different from those initially present in the patients' urine samples. This is clearly exemplified by sample 3, which contained flurazepam (in addition to clorazepate) and was identified from the mass spectra for the N-desalkylflurazepam (Fig. 3), where the molecular ion and the loss of a fluorine atom are apparent. The chromatograms for the benzophenones found in urine

Table 3
Application of the screening and confirmatory GC–MS methods to urine samples

Urine	Drug administered	Screening response	GC–MS confirmation (μM)
1	None	Negative	–
2	Clorazepate	Positive	8.1±0.4
3	Clorazepate	Positive	6.5±0.3
	Flurazepam		N-desalkylflurazepam detected
4	Lorazepam	Positive	10.3±0.6
	Acetaminophen		
5	Alprazolam	Positive	1.5±0.1
	Flunitrazepam		1.1±0.1
6	Ketazolam	Positive	12.5±0.6
	Lormetazepam		6.0±0.4
	Lorazepam		6.0±0.4
7	Alprazolam	Positive	2.3±0.1
	Lormetazepam		1.0±0.1
8	Alprazolam	Positive	3.4±0.2
9	Alprazolam	Positive	5.3±0.3

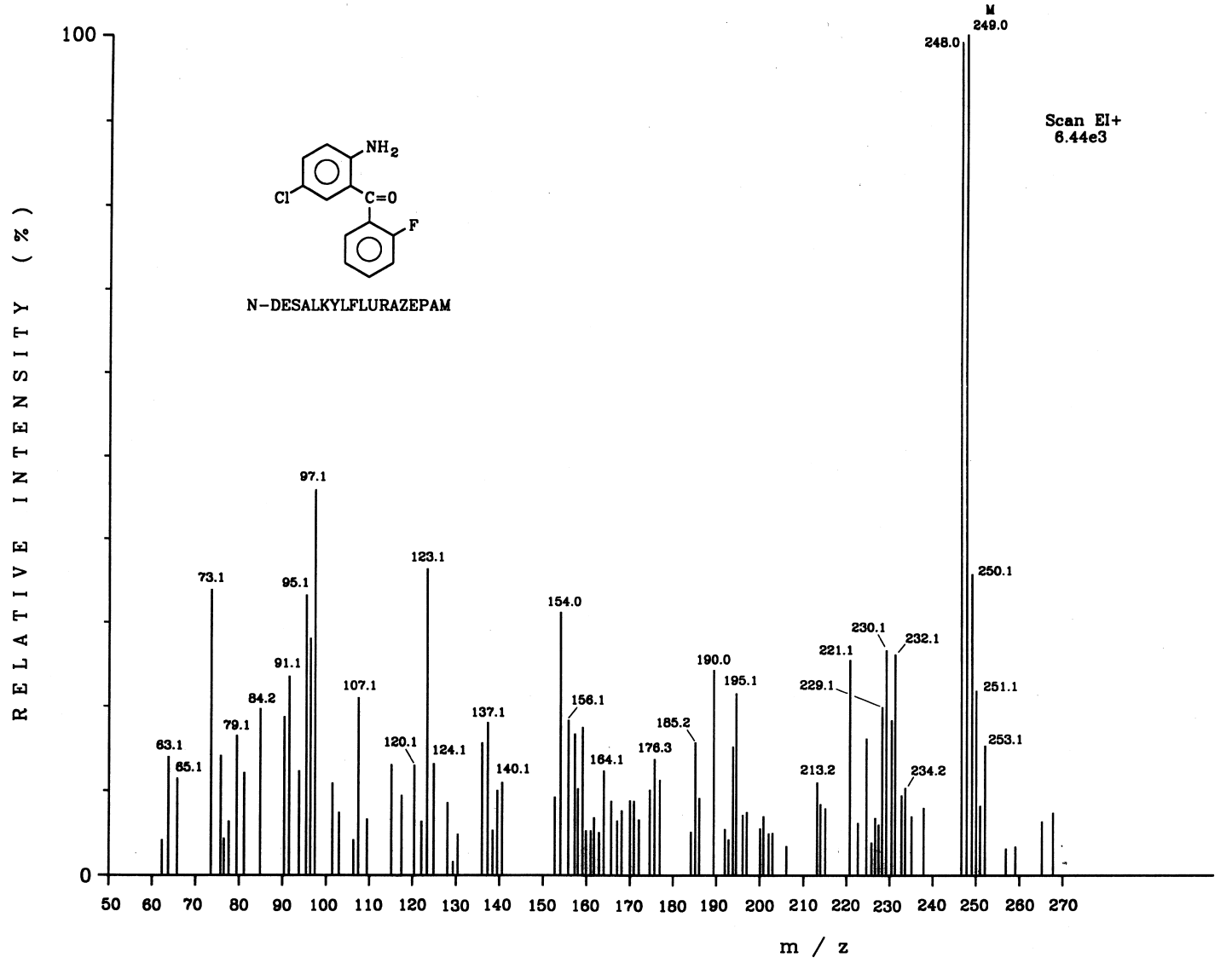

Fig. 3. Mass spectra for N-desalkylflurazepam detected in urine 3 (see Table 3) after the GC–MS confirmatory method; $m/z=249$ corresponds to the molecular ion; $m/z=230$ is the loss of a fluorine atom.

6 at the m/z values selected for identification are shown in Fig. 4. As can be seen, the benzophenone of oxazepam was obtained because ketazolam is excreted as oxazepam (56% of the dose [15]); also, the benzophenones of lormetazepam and lorazepam were readily detected (see Fig. 4).

4. Conclusions

The proposed methods works in two ways: one is by cleaving glucuronide bonds and producing more free benzodiazepines; the other is by converting benzodiazepines into benzophenones. Therefore, be-

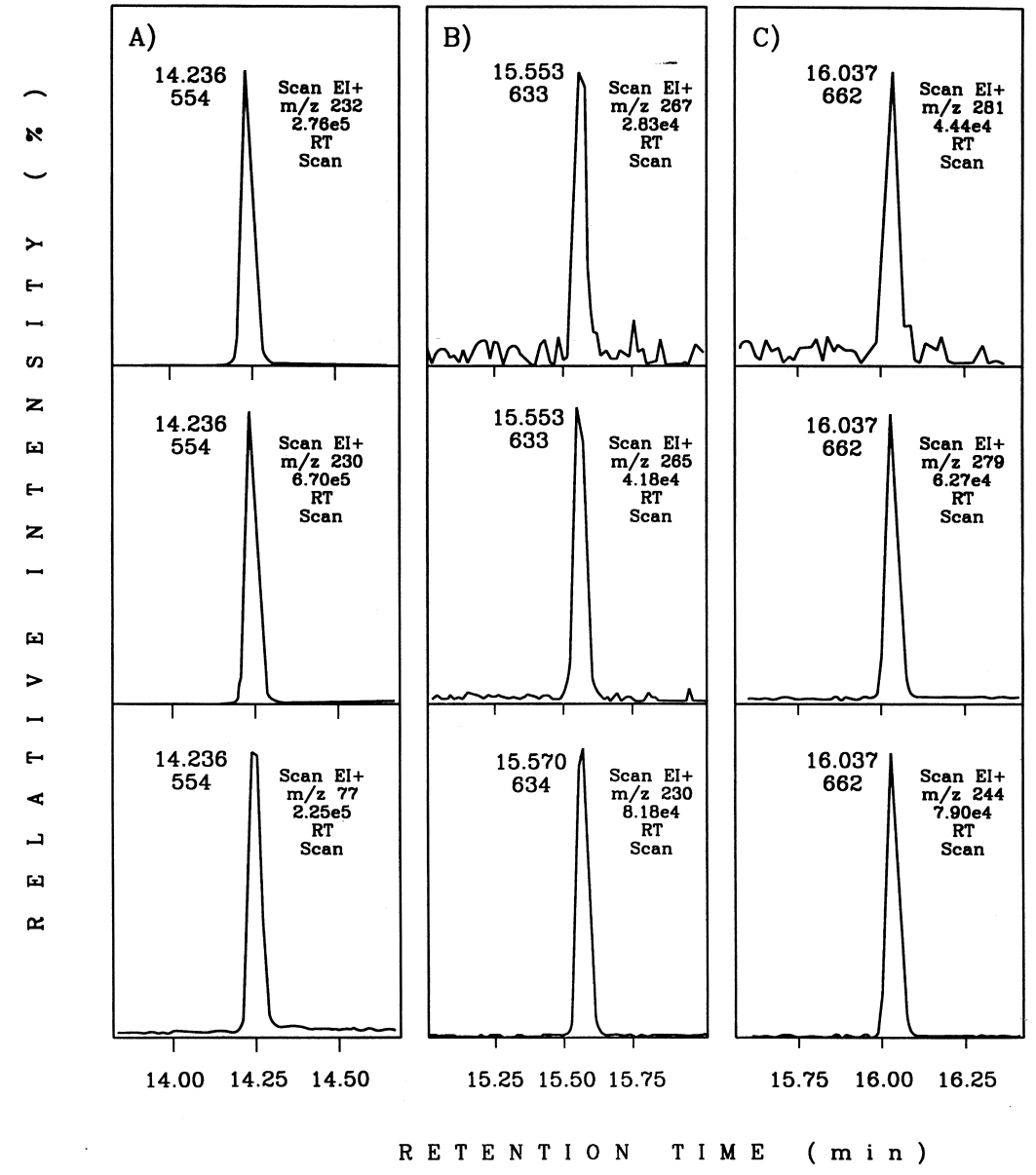

Fig. 4. TIC and m/z chromatograms for oxazepam, from ketazolam (A), lorazepam (B) and lormetazepam benzophenones (C) found in urine sample 6 (Table 3). The three m/z values shown are those selected for confirmation/identification and quantitation.

cause benzodiazepines are often found as glucuronide conjugates, the sensitivity is boosted as a result. The thermal instability of some of these drugs, particularly oxazepam, which complicates their chromatographic determination, [16] is avoided by using benzophenones, which are more stable than the parent compounds (oxazepam, lorazepam) and dispense with the need for derivatization to increase the thermal stability of the drugs (e.g. N-methyl or trimethylsilyl [2,11] derivatives). In most cases, the benzodiazepine consumed can be determined via the benzophenone detected; in some cases this is the sole available choice (e.g. alprazolam is directly metabolized to its benzophenone). Finally, hydrolysis of the urine samples is the more time consuming-step; however, it is also required in other screening and GC–MS methods [6,9–12] and allows simultaneously processing of a large number of samples. The effective hydrolysis time for the proposed screening method is 1 h as opposed to 2 h in immunoassay; in addition, is more selective and cost-effective than immunochemical methods.

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