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## MIDAZOLAM DETERMINATION BY GAS CHROMATOGRAPHY, LIQUID CHROMATOGRAPHY AND GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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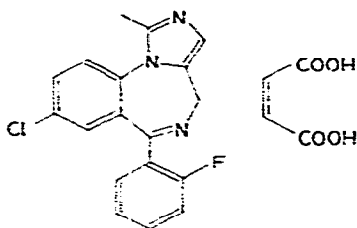
### SUMMARY

Midazolam is determined in serum by gas chromatography with a nitrogen-selective detector, by liquid chromatography, and by gas chromatography—mass spectrometry. Comparable results are obtained with the three techniques with a within-run precision of 9% by gas chromatography and gas chromatography—mass spectrometry and 5% by liquid chromatography. Between-run precision is 13% by gas chromatography—mass spectrometry and 10% by liquid chromatography. Comparison of patient's sera by gas chromatography ( $x$ ), liquid chromatography ( $y$ ), and by gas chromatography ( $x$ ), gas chromatography—mass spectrometry ( $y$ ) gave correlations of 0.98 and 0.89, respectively. Interferences observed when using one technique, for example liquid chromatography, can be eliminated by analyzing the sample extract with one of the other techniques.

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### INTRODUCTION

Midazolam maleate (I) is an investigational benzodiazepine which is pharmacologically similar to diazepam. Because of its shorter half-life, which is less than three hours, and greater water solubility, midazolam is especially suited for induction of anesthesia [1–4]. Therapeutic and toxic midazolam concentrations have not yet been established for humans; however, since midazolam is twice as potent as diazepam, therapeutic response and toxicity would be expected to occur at lower blood concentrations [1, 3].



MIDAZOLAM MALEATE (I)

Gas chromatographic (GC) procedures, for the determination of midazolam in serum, with the use of an electron-capture detector, and a nitrogen-selective detector have been reported [2, 4–6]. More recently, we reported a liquid chromatographic (LC) procedure for the determination of midazolam and its 1-hydroxymethyl metabolite in serum [7].

In this report we have included gas chromatography—mass spectrometry (GC—MS) in the analytical midazolam procedure and the use of prazepam as internal standard for LC. The result of the above modification was to make our midazolam procedure more sensitive and selective.

## MATERIALS AND METHODS

### Apparatus

A Model 5840A gas chromatograph with dual nitrogen—phosphorus-sensitive detectors and coiled glass columns (1.2 m × 2 mm I.D.) packed with 2% SP-2250 on Chromosorb W HP (100–120 mesh) (Hewlett-Packard, Avondale, PA, U.S.A.) was used.

A Model 6000A solvent delivery system, Model 600 solvent programmer, Model 440 absorbance detector, Model 450 variable-wavelength detector, Model U6K universal liquid injector (Waters Assoc., Milford, MA, U.S.A.), and a dual-pen recorder (Houston Instruments, Austin, TX, U.S.A.) were used for LC separations. We also used a prepacked 10- $\mu$ m particle size  $\mu$ Bondapak C<sub>18</sub> (300 × 4 mm I.D.) column from Waters Assoc.

GC—MS was performed on a Model 5985A quadrupole system (Hewlett-Packard) with selective ion monitoring (SIM), and electron impact. The system consisted of a Hewlett-Packard 5840A gas chromatograph interfaced to the mass spectrometer. The glass column was 1.2 m × 2 mm I.D. packed with 2% SP-2250 Chromosorb W HP (100–120 mesh). The ionization energy was  $112.14 \cdot 10^{-19}$  J (70 eV), the electron multiplier voltage was set at 3000 V, and the dwell time for each ion monitored was 100 msec.

### Reagents

Heptane, diethyl ether and isobutanol were analytical reagent (AR) grade. Anhydrous sodium sulfate was used. High-purity methanol was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Sodium hydroxide, 0.5 mol/l and 4.4 mol/l was prepared from a concentrated solution. Sulfuric acid, 1 mol/l and 0.05 mol/l was prepared from a concentrated solution.

Octanesulfonic acid, 0.05 and 0.005 mol/l, was prepared from the sodium

salt which was obtained from Eastman Organic Chemicals (Rochester, NY, U.S.A.).

**LC mobile phase.** The LC mobile phase consisted of 0.005 mol/l octanesulfonic acid in methanol. Dilute 50 ml of 0.05 mol/l octanesulfonic acid with 150 ml of water and dilute to 500 ml with methanol. Adjust the pH of this mixture to 4.0 with 0.05 mol/l sulfuric acid.

### **Standards**

Midazolam maleate, 1-hydroxymethylmidazolam, desmethylmidazolam and flurazepam dihydrochloride were obtained from Hoffmann-La Roche (Nutley, NJ, U.S.A.). Prazepam was obtained from Warner-Lambert Research Institute (Morris Plains, NJ, U.S.A.). Standards of midazolam, its metabolites, and the internal standards flurazepam or prazepam were prepared at 1 g/l in absolute ethanol. Dilutions of the 1 g/l solutions with water were used to make the working (0.05–2.0 mg/l) standards of midazolam and its metabolites in water. Serum standards were prepared by reconstituting lyophilized human serum with the aqueous standards. Solutions of the internal standards, flurazepam and prazepam (5 mg/l) were prepared from the 1 g/l ethanol stock in water.

### **Operating conditions**

For GC analysis, we used a column packed with 2% SP-2250 at 260°C and a helium flow-rate of 40 ml/min. For LC we used a C<sub>18</sub> reversed-phase column and a flow-rate of 1.0 or 1.5 ml/min with a mixture of methanol–water (60:40, v/v) as the mobile phase. The eluent was monitored at 254 and 220 nm with the use of constant- and variable-wavelength detectors which were in series.

For GC–MS, we used a 2% SP-2250 column at 250°C with a helium flow-rate of 40 ml/min. Masses monitored were 310 and 325 for midazolam and 58, 86 or 183 for flurazepam.

### **Procedure**

The extraction procedure has been reported previously [4, 7]. To 1–2 ml of serum was added 0.2 ml of 5 mg/l internal standard (flurazepam or prazepam). The serum was made basic with 4 ml of 0.5 mol/l sodium hydroxide and was then extracted into 25 ml of heptane–isobutanol (96:4, v/v). The sample was back-extracted into 4 ml of 1 mol/l sulfuric acid, which was then made basic with 2.5 ml of 4.4 mol/l sodium hydroxide and was back-extracted into 10 ml of diethyl ether. The ether was dried by passing it through anhydrous sodium sulfate and was then evaporated to dryness under nitrogen. The residue was dissolved in 25  $\mu$ l of absolute ethanol and 1 or 2  $\mu$ l was injected for GC or GC–MS analysis. For LC the residue was dissolved into 50  $\mu$ l of absolute ethanol and 20  $\mu$ l were injected for LC analysis.

We used the ratios of the peak heights of midazolam to that of the internal standard, flurazepam or prazepam, to calculate concentration. For GC–MS analysis, the ratios of the area counts at *m/e* 325/58, 310/58, 325/86 or 310/86 were used to calculate the midazolam concentration.

## RESULTS

*Liquid chromatography*

Chromatograms from LC of a serum blank, and serum standards of 250 and 500  $\mu\text{g/l}$  at 220 nm are given in Fig. 1. No interference was observed from sera blanks that were assayed by this procedure in the region where midazolam and 1-hydroxymethylmidazolam are eluting. Fig. 2 shows serial analysis of a patient's serum after infusion with midazolam. The relative retention times for 1-hydroxymethylmidazolam and midazolam are 0.40 and 0.50, respectively, relative to prazepam which elutes at 17 min. Similar results are observed at 254 nm. When peak heights of sera containing 0.25, 0.5, 1 and 2 mg/l of midazolam and 1-hydroxymethylmidazolam were plotted against concentration, the resulting lines had a slope of 0.0026,  $y$ -intercept of  $-0.56$ , standard error of estimate ( $S_{yx}$ ) of 0.098, correlation coefficient ( $r$ ) of 0.99 for 1-hydroxymethylmidazolam and 0.0035,  $-0.145$ , 0.067 and 0.99, respectively, for midazolam. Within-run precision averaged 5 and 8% for midazolam and 4 and 5% for 1-hydroxymethylmidazolam at the 0.5 and 1 mg/l concentrations, respectively ( $n = 3$  in each case). Linearity of the procedure extended to less than 0.1 mg/l using the present procedure. This was demonstrated by extracting 1 ml of a 100  $\mu\text{g/l}$  serum standard which was reconstituted to 50  $\mu\text{l}$  with ethanol and injecting 20  $\mu\text{l}$  of the ethanol for analysis (0.02 a.u.f.s.). The sensitivity of the method is, therefore, calculated to be 15  $\mu\text{g/l}$  using 2 ml of serum, for a peak height ratio of twice background. Diazepam interferes with midazolam while nordiazepam interferes with 1-hydroxymethylmidazolam, as previously reported [7].

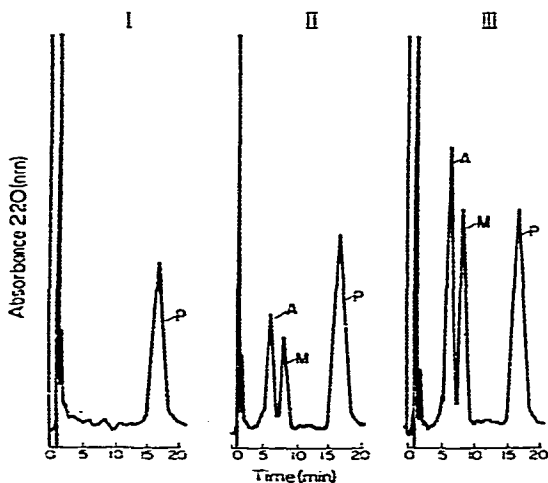


Fig. 1. Liquid chromatograms of a serum blank (I); and serum standards containing 250 (II), and 500 (III),  $\mu\text{g/l}$  of midazolam and 1-hydroxymethylmidazolam. Peaks: A = 1-hydroxymethylmidazolam, M = midazolam, P = prazepam (internal standard, 500  $\mu\text{g/l}$ ). Conditions: 0.02 a.u.f.s.; 20  $\mu\text{l}$  injected; eluting solvent methanol-water (60:40, v/v) with octanesulfonic acid for ion-pairing, pH 4.0; flow-rate, 1.5 ml/min; absorbance, 220 nm; 1 ml serum used for analysis.

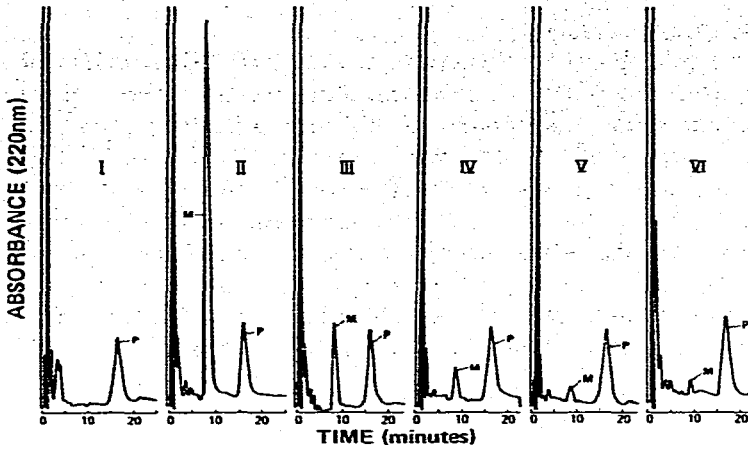


Fig. 2. Liquid chromatograms of a patient's sera midazolam concentrations taken at specified time intervals post induction, I = blank, time = 0; II = 1520  $\mu\text{g/l}$ , time = 1 min; III = 370  $\mu\text{g/l}$ , time = 2.5 min; IV = 185  $\mu\text{g/l}$ , time = 15 min; V = 111  $\mu\text{g/l}$ , time = 45 min; VI = 96  $\mu\text{g/l}$ , time = 3 h. Absorbance, 220 nm; 0.02 a.u.f.s.; flow-rate, 1.5 ml/min; methanol-water, (60:40, v/v) with octanesulfonic acid, pH 4.0. Peaks: M = midazolam, P = prazepam (internal standard, 500  $\mu\text{g/l}$ ).

#### Liquid and gas chromatography compared

Sera from 22 patients who were induced for anesthesia with midazolam were first analyzed by GC and then by LC. For this comparison flurazepam was used as internal standard since prazepam interferes with midazolam by GC. The results are illustrated in Fig. 3. Statistical analysis, GC ( $x$ ) and LC ( $y$ ), gave a slope of 1.09,  $y$ -intercept of 120, a standard error of estimate ( $S_{yx}$ ) of 111, and a correlation coefficient of 0.98 [7].

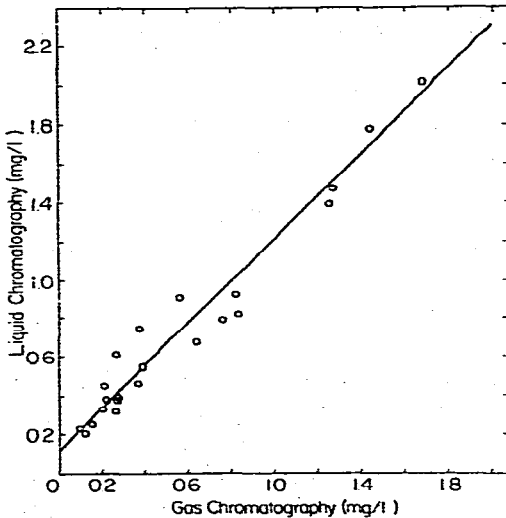


Fig. 3. Comparison of determinations of midazolam in patients' sera by GC with a nitrogen-selective detector and by LC. Internal standard = flurazepam.

### Gas chromatography—mass spectrometry

When peak area ratios of midazolam to flurazepam,  $m/e$  310/86 and 310/58, of sera containing 50, 100, 200 and 500  $\mu\text{g/l}$  of midazolam were plotted against concentration the resulting lines had a slope of 0.0007 and 0.0185,  $y$ -intercept of  $-0.081$  and  $0.0392$ , standard error of estimate ( $S_{yx}$ ) of 0.011 and 0.274, respectively, and correlation coefficients ( $r$ ) of 0.996. Similar results were obtained in using the mass ratio 325/58 for calculating the concentration of midazolam in serum. Within-run precision (coefficient of variation) of a serum based control ( $n = 4$ ) was 9% while between-run precision was 7% ( $n = 6$ ). A comparison of determinations of ten patients' samples GC ( $x$ ) and GC-MS ( $y$ ) (0.2–2.0  $\text{mg/l}$ ) resulted in a line with a slope of 0.622, a  $y$ -intercept of 0.23,  $S_{yx}$  of 0.14 and correlation ( $r$ ) of 0.89.

### DISCUSSION

Midazolam maleate is an experimental benzodiazepine which is not currently available for routine clinical use. As part of an experimental protocol in evaluating the drug for induction of anesthesia, we developed the present extraction procedure coupled with GC, LC and GC-MS for its determination in serum and urine. Midazolam has been determined by GC using an electron-capture and a nitrogen-selective detector [2, 4–6]. Using a 3% OV-1 column at 225°C midazolam has been reported to have a retention time of 5.8 min using nitrogen (flow-rate, 65 ml/min) as carrier gas [2]. Under our conditions of study, with the use of a more polar 2% SP-2250 column, at 260°C and 40 ml/min helium flow-rate, midazolam has a relative retention time of 0.46 to flurazepam (2.1 min).

The major reported metabolite of midazolam is 1-hydroxymethylmidazolam [1, 3, 6]. It does not chromatograph well on a GC column and cannot, therefore, be quantitated by GC without derivatization [2, 6]. In order to detect the presence of the 1-hydroxy metabolite in addition to other possible metabolites in serum, we developed an LC procedure for the determination of midazolam and its metabolite. Using the present extraction procedure, *in vitro* samples of midazolam and its 1-hydroxymethylmetabolite are separated using methanol–water (60:40, v/v). In order to have a more sensitive and selective procedure for the determination of midazolam than GC or LC, we used GC-MS with the presence of a non isotopically labelled internal standard for the identification and confirmation of midazolam.

Using the present extraction procedure the absolute recovery averaged  $74 \pm 6\%$  ( $n = 5$ ) for midazolam using serum extracts of 0.75–5  $\text{mg/l}$ . The absolute recovery for the 1-hydroxymethyl metabolite averaged  $97 \pm 5\%$  ( $n = 5$ ). With use of serum standards the relative percent recovery was 100% for both midazolam and its 1-hydroxy metabolite [4, 7]. Within-run precision was 9% by GC and GC-MS and 5% by LC at the 500  $\mu\text{g/l}$  concentration, while between-run precision was 13% by GC ( $n = 13$ ), 9% ( $n = 6$ ) by GC-MS, and 10% ( $n = 5$ ) by LC at the 500  $\mu\text{g/l}$  concentration. In the same concentration range, GC, LC and GC-MS, therefore, give comparable results. For the routine determination of midazolam in the  $\text{mg/l}$  range, GC has a distinct advantage over GC-MS and LC since it is faster than LC and much simpler instrumentation is required than for

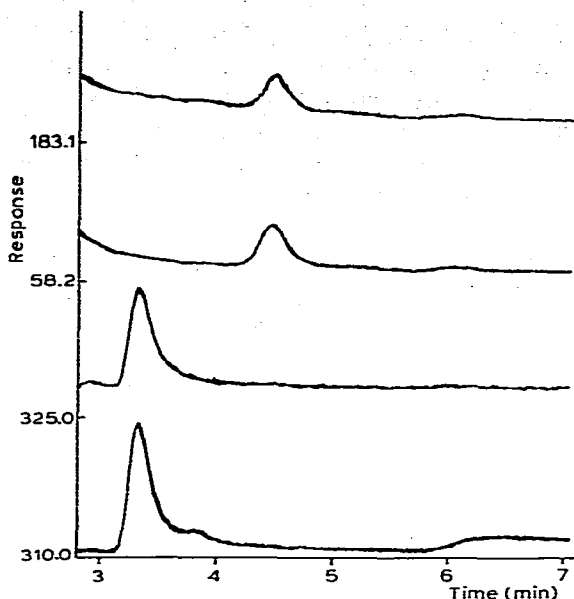


Fig. 4. GC-MS of a patient's urine extract using SIM. Concentration of midazolam is 71  $\mu\text{g/l}$ . Masses  $m/e$  310 and 325 are for midazolam and  $m/e$  58 and 183 are for flurazepam. Conditions: electron-impact (EI) mode. Two-percent SP-2250 column, 250°C. Flurazepam internal standard (40  $\mu\text{g/l}$ ).

GC-MS. The advantage of GC-MS over GC is in the  $\mu\text{g/l}$  range where specificity and sensitivity are needed for identification. Fig. 4 shows a GC-MS spectrum using the selected ion mode (SIM) of urine from a patient given midazolam. The presence of midazolam at very low concentration is evident in Fig. 4. The presence of the 1-hydroxy metabolite could not be determined since it does not chromatograph on a GC column. Masses monitored are 310, 325 for midazolam and 58, 183 for flurazepam. The amount of midazolam which was excreted in the urine was less than 1% of the total dose given. In order to see the presence of the drug in Fig. 4, 25 ml of a 24-h urine was extracted and analyzed by GC-MS. The same sample analyzed by GC with a nitrogen-selective detector did not give an unequivocal identification of the presence of midazolam. The above example, therefore, illustrates the selectivity and sensitivity of GC-MS over GC. The same advantage of specificity is also true for GC-MS over LC.

GC and GC-MS are limited in their utility for the direct determination of midazolam since underivatized 1-hydroxymethylmidazolam does not chromatograph well on a GC column. More recently, a desmethyl metabolite of midazolam has been identified which may be of clinical importance. Desmethylmidazolam has the same retention time as midazolam on a 2% SP-2250 column and cannot be separated by GC on this column, or the less polar 3% OV-1 column. Its mass spectrum has fragments at  $m/e$  311, 283, 91 and 313. The fact that the desmethyl metabolite has the same retention time as midazolam, and 1-hydroxymethylmidazolam does not chromatograph on a GC column, therefore, gives LC a distinct advantage over GC and GC-MS for the determination of parent drug and metabolites. Using a methanol-water (60:40, v/v) mixture

with ion-pairing, midazolam, 1-hydroxymethylmidazolam and desmethyldiazepam are readily separated. At a flow-rate of 1.0 ml/min and pH 4.0, retention times are 0.67, 0.79, and 0.88 for 1-hydroxymethylmidazolam, desmethyldiazepam and midazolam, respectively, relative to prazepam. Our preliminary data indicate that no appreciable amounts of free 1-hydroxymethylmidazolam and desmethyldiazepam are observed in sera of patients who were induced for anesthesia with intravenous midazolam (Fig. 2). The conjugated form of 1-hydroxymethylmidazolam has been reported in urine, which suggests that 1-hydroxymethylmidazolam is quickly cleared from the circulatory system by conjugation [2]. We have not seen the presence of desmethyldiazepam or 1-hydroxymethylmidazolam by LC in sera up to 24 h post induction. In contrast, 1-hydroxymethylmidazolam has been reported in a patient who was given a 10-mg oral dose of midazolam [6].

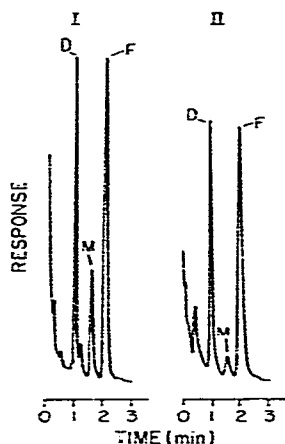


Fig. 5. Gas chromatogram of a serum extract with 3 mg/l of diazepam and midazolam (I) and a patient's serum with 3.70 mg/l diazepam and 0.4 mg/l midazolam. Peaks: D = diazepam, M = midazolam, F = flurazepam (internal standard, 2.6 mg/l). Conditions: 2% SP-2250 column at 260°C.

Interference studies indicate that diazepam interferes with midazolam by LC [7]. By GC, however, both drugs are separated and can be quantitated with flurazepam as internal standard (Fig. 5). Thus, there is a distinct advantage of GC over LC for the determination of midazolam in serum in the presence of diazepam (Fig. 5). In Fig. 5 we see the determination of diazepam in the presence of midazolam which could not be performed with the present LC procedure. Quinidine, methaqualone and oxazepam will interfere with 1-hydroxymethylmidazolam by LC. The following drugs were shown not to interfere by GC: oxazepam, chlordiazepoxide, norchlordiazepoxide, diazepam, amitriptyline, nortriptyline, doxepin, desmethyldoxepin, imipramine, desipramine, quinidine, procainamide and disopyramide [4]. By LC the following drugs will not interfere: amitriptyline, nortriptyline, doxepin, imipramine, procainamide, loxapine, phenobarbital, secobarbital, salicylate, phenytoin, meprobamate, glutethimide and disopyramide. Flurazepam was initially chosen as internal standard in order to have the flexibility of performing the analysis by GC, LC and GC-MS. If one wishes to use only LC, prazepam is a preferred internal



standard, since it is better resolved from the midazolam metabolites than is flurazepam. The use of either internal standard gives comparable results for the analysis of midazolam. An advantage of using flurazepam as an internal standard is that interference from quinidine, oxazepam and methaqualone which may be present in serum and interfere with the LC method can be overcome by performing a GC analysis.

Interferences in chromatographic procedures by drugs which are used in the clinical laboratory is an ever present problem which we all confront every day. One solution to this problem is to change the pH of extraction (chemical technique) or to change the chromatographic technique which is being used. A more rational approach in dealing with interference problems, is to set up the analytical procedure whereby at the end of the sample preparation, different chromatographic techniques, such as GC, LC or GC-MS can be used for the analysis. The choice of which chromatographic technique is used depends on the analytical problem to be solved. This may include interference problems, or the quantitation of metabolites. This we feel is a more fruitful approach to the analytical problem at hand than to use one technique because of convenience or fashion.

The use of GC, LC and GC-MS has been demonstrated for the determination of midazolam in serum. Correlation studies which were performed, demonstrate that the three techniques give comparable results for its determination. For the determination of parent drug and metabolites, LC has a distinct advantage over GC and GC-MS, since they can be measured directly without any derivatization. Desmethylmidazolam and 1-hydroxymethylmidazolam were not seen in serum by LC, although we did not look for these metabolites in patient's urines. Sensitivity of the GC method is 50  $\mu\text{g/l}$  while LC and GC-MS have sensitivities of 15 and 2  $\mu\text{g/l}$ , respectively.

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