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QUANTITATIVE DETERMINATION OF CLONAZEPAM AND ITS METABOLITES IN HUMAN PLASMA BY GAS CHROMATOGRAPHY

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SUMMARY

A method for the determination of clonazepam (Rivotril®) and its two main metabolites in plasma by gas chromatography has been developed.

The three compounds and an added internal standard were extracted from plasma with ethyl acetate. The subsequent steps included evaporation, purification and differential extraction of clonazepam and the metabolites. A gas chromatograph equipped with an electron capture detector was used.

The limit of sensitivity is 3–5 ng/ml, which is sufficient to determine concentrations in plasma from patients being treated with clonazepam.

The specificity of the method was confirmed by mass fragmentography.

INTRODUCTION

The value of reliable kinetic information on anti-epileptic drugs in humans is evident from experience with phenytoin, carbamazepine and phenobarbital[1]. Clonazepam is a relatively new anti-epileptic agent, and most pharmacokinetic information about it has been based on results from single-dose experiments with ^{14}C-labelled clonazepam[2]. Only limited work has been carried out on the determination of its concentrations in plasma in patients undergoing continuous treatment with clonazepam.

A method for the determination of clonazepam in plasma based on gas chromatography (GC) with electron capture detection (ECD) has been described earlier[3]. However, this method later proved to co-determine part of the main metabolite of clonazepam. The result would therefore be the sum of the concentrations of clonazepam and about 10% of the main metabolite (measured as nanogram equivalents of clonazepam per millilitre).

Some other GC methods for the determination of clonazepam are being developed in various laboratories. These procedures are based on the measurement of the benzophenone produced by acid hydrolysis of clonazepam and are somewhat

analogous to the determination of diazepam as described by De Silva *et al.*[4]. No other method has been published to our knowledge.

The purpose of the present investigation was to develop a method by which clonazepam and its metabolites (Fig. 1) could be determined in plasma in order to correlate the plasma levels with the efficacy and side-effects of the treatment.

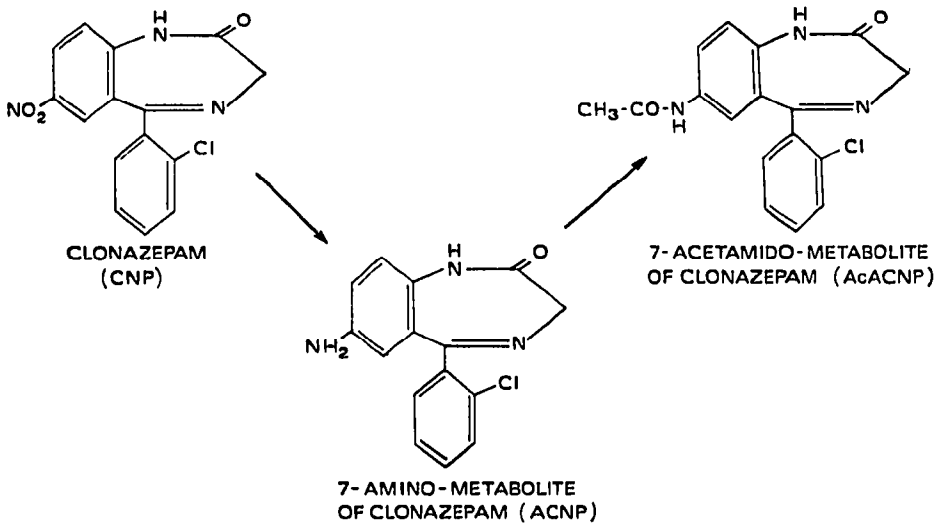


Fig. 1. Clonazepam and its metabolites present in human plasma according to Eschenhof[2].

In order to test the specificity of the method, a combined GC–mass spectrometric technique (mass fragmentography[5,6]) was utilized. This technique has been used for the quantitative determination of, *e.g.*, the antidepressant drug nortriptyline and its metabolites in plasma[7].

By this method, characteristic ions of the mass spectrum of material with the chromatographic retention time of the reference compound were recorded and their intensities were used as reference for quantitative measurements.

EXPERIMENTAL

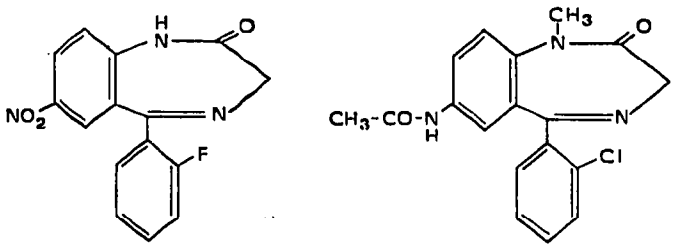
Reagents, solvents and glassware

n-Hexane and toluene of low grades of purity were distilled once before use. Ethyl acetate and methanol (both from E. Merck, Darmstadt, G.F.R.) were of analytical-reagent grade.

Glassware was cleaned with detergent in an ultrasonic bath and rinsed twice with redistilled water and once with methanol. The glassware that came into contact only with organic solvents was wetted with methanol before use so as to prevent adsorption of the compounds being determined.

Reference substances

Structural formulae are shown in Figs. 1 and 2.



Ro 5-44 35
I.S. (1)

Acetylated Ro 10-33 84
I.S. (2)

Fig. 2. Structural formulae of the two internal standards.

Clonazepam (CNP), the 7-amino metabolite (ACNP), the 7-acetamido metabolite (AcACNP) and Ro 5-4435 (internal standard for clonazepam) were donated by Roche, Basle, Switzerland.

The internal standard for the metabolites was prepared by acetylation of Ro 10-3384 with a solution of acetic anhydride in toluene (10 min at 90°).

Samples for standard curves

Known amounts of the drug and metabolites were added in increasing concentrations to plasma blanks.

For clonazepam and the amino metabolite, standard curves from 15 to 100 ng/ml were used, and for the acetamido metabolite a standard curve from 4 to 25 ng/ml was used.

Extraction procedure

To 2 ml plasma in a centrifuge tube was added 150 ng of the first internal standard. The sample was mixed for 5 min with 4 ml of ethyl acetate by rotation. After centrifugation, 2.5 ml of the organic phase were transferred into a 10-ml glass-stoppered tube and evaporated under a stream of nitrogen. The residue was dissolved in 2 ml of 0.01 *N* hydrochloric acid and washed twice with 4 ml of *n*-hexane for 3 min. The *n*-hexane phase was discarded. Clonazepam and the internal standard, but not the metabolites, were extracted with 4 ml of toluene by mixing for 5 min. The toluene phase was transferred into a tapered tube and evaporated, and the residue dissolved in 100 μl of toluene. About 1.5 μl of this solution was injected into the gas chromatograph for the determination of clonazepam.

The aqueous phase was washed twice with 4 ml of toluene for 3 min. The toluene phase was discarded. An amount of 50 ng of the second internal standard was added and the solution was buffered by adding 0.2 ml of 1 *M* phosphate buffer of pH 7.4. The metabolites and the internal standard were extracted with 4 ml of a mixture of toluene and ethyl acetate (1:1) by mixing for 5 min. The organic phase was transferred into a tapered tube and evaporated, and the residue dissolved in 50 μl of toluene. About 1.5 μl of this solution was injected into the gas chromatograph for the determination of the metabolites.

Determination of clonazepam as its hydrolytic product (benzophenone derivative, Fig. 3)

The extraction procedure was performed as described above. The extract was

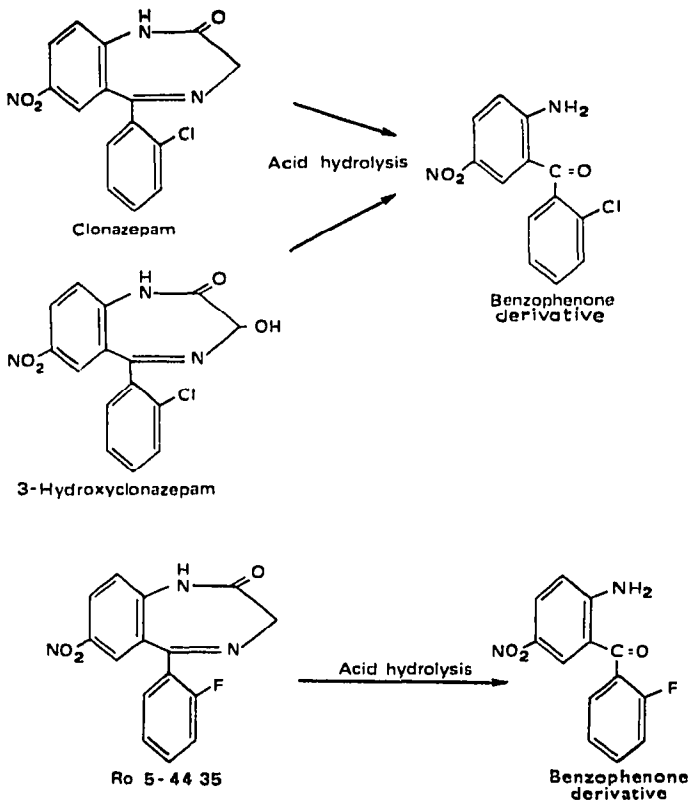


Fig. 3. Benzophenones formed by acid hydrolysis of clonazepam, 3-hydroxyclonazepam and Ro 5-4435.

then evaporated and hydrolyzed in 1 *N* sulphuric acid for 2 h. The acid phase was made alkaline with 300 μl of 4 *N* sodium hydroxide solution and the benzophenones were extracted with 4 ml of *n*-hexane. The *n*-hexane phase was transferred into a tapered tube and evaporated and the residue was dissolved in *n*-hexane and used for GC and mass fragmentography.

Extraction recoveries

Extraction recoveries were assessed by extraction of microgram amounts of the various compounds from aqueous phases at different pH values with various extraction media.

The extracts were measured on a gas chromatograph equipped with a flame ionization detector. The results are shown in Table I.

Gas chromatography

The analyses were performed on a Pye Series 104 gas chromatograph equipped with an ECD. The conditions used for GC were as follows. Column: glass, 0.9 m long, 4 mm I.D.; 1% OV-17 on Celite J.J. CQ, 100–120 mesh. Temperatures: pre-heater, 270°; detector oven, 350°; column oven, 270°, 280° and 240° for the determination of

clonazepam, the metabolites and the benzophenones, respectively. Carrier gas: argon–methane (90:10) at a flow-rate of 80 ml/min. Pulse interval: 50 μsec. Attenuation: 5×10^2. The column was deactivated by silylation with hexamethyldisilazane and injection of ethyl acetate extracts of blank plasma.

Mass spectrometry–mass fragmentography

A combined LKB 9000 gas chromatograph–mass spectrometer was used. The experimental conditions used for the mass spectrometry were as follows. Ionization energy, 70 eV. Trap current, 60 μA. Accelerating voltage, 3500 V.

The mass spectra of clonazepam and of the amino metabolite are shown in Fig. 4. The base peak at *m/e* 280 and the peak of the molecular ion of *m/e* 315 of clonazepam, and the peak at *m/e* 285, which is both the base peak and represents the molecular ion of the amino metabolite, were used for mass fragmentographic measurement.

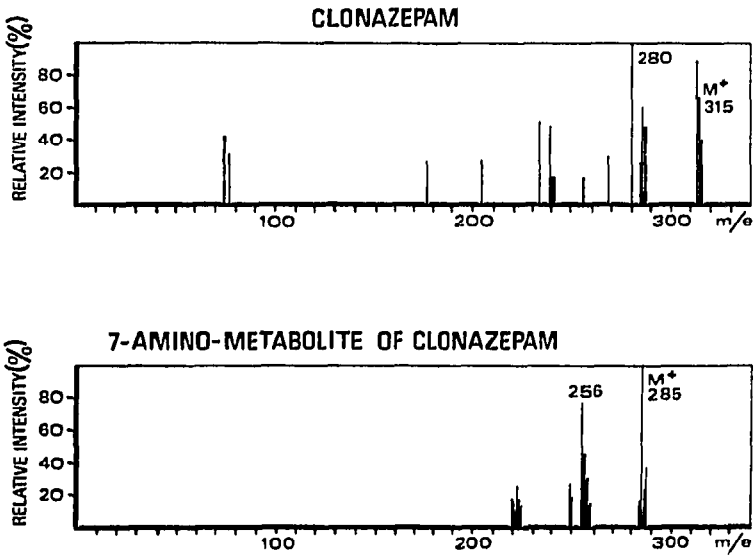


Fig. 4. Mass spectra of clonazepam and its 7-amino metabolite.

The hydrolyzates of clonazepam and of the first internal standard were measured by mass fragmentography. The molecular ions at *m/e* 276 and 260 were used for the determination.

RESULTS AND DISCUSSION

Extraction

The extraction properties of clonazepam, its metabolites and some related benzodiazepines are shown in Table I.

As benzodiazepines might be extensively bound to plasma proteins, an efficient medium for the extraction of benzodiazepines from plasma is desirable. For diazepam[8] and nitrazepam[9], the degrees of protein binding were found to be 96 % and

TABLE I

EXTRACTION OF CLONAZEPAM, ITS METABOLITES AND SOME RELATED BENZODIAZEPINES WITH DIFFERENT ORGANIC SOLVENTS FROM EQUAL VOLUMES OF AQUEOUS PHASES AT DIFFERENT pH VALUES

The figures in the table show the percentage of the benzodiazepine extracted into the organic phase.

Compound	*Aqueous phase*					
	Buffer, pH 7.4	*0.01 N HCl*	*Buffer, pH 7.4*	*0.01 N HCl*	*Buffer, pH 7.4*	*0.01 N HCl*
	Organic phase					
	Ethyl acetate	*n-Hexane*	*Toluene*	*Toluene*	*Toluene–ethyl acetate (1:1)*	*Toluene–ethyl acetate (1:1)*
Clonazepam	~100	~0	97	90	99	97
Ro 5-4435	~100	~0	91	70	96	88
7-Amino metabolite of clonazepam	~100	~0	36	< 2	85	7
7-Acetamido metabolite of clonazepam	93	~0	7	< 2	78	37
Acetylated Ro 10-3384	~100	~0	68	36	96	82

86%, respectively. Ethyl acetate was the most efficient extraction medium tried and consequently it was the most suitable for the extraction of the benzodiazepines from plasma.

In the following steps of the procedure, less efficient extraction media have to be used in order both to separate clonazepam and the metabolites into different extract fractions and to eliminate impurities extracted from the plasma with ethyl acetate.

Washing of aqueous phases containing clonazepam could be carried out only with organic solvents with very low polarities, *e.g.*, *n*-hexane, otherwise there would be a large loss of the compound. The use of very strong acids to keep the benzodiazepines in the aqueous phases was avoided because of the risk of hydrolysis of clonazepam.

Formation of benzophenones by hydrolysis of benzodiazepines

Under the hydrolytic conditions described, the disappearance of clonazepam and of Ro 5-4435 was total, and the formation of the corresponding benzophenones seemed to be quantitative; at least it was reproducible.

Plasma analysis

Typical chromatograms of blank plasma and plasma from a patient treated with clonazepam are shown in Figs. 5, 6 and 7.

The simultaneous determination of clonazepam and the metabolites without making a differential extraction was not possible because of the poor chromatographic separation of clonazepam and the amino metabolite (see Fig. 8). Other column materials (OV-1, OV-101 and Dexsil) were tried, but did not give better separations. Changes in column length, support or percentage of loading were not tried, as we

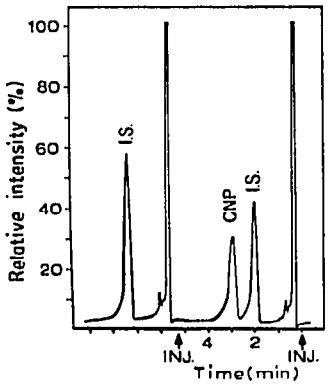
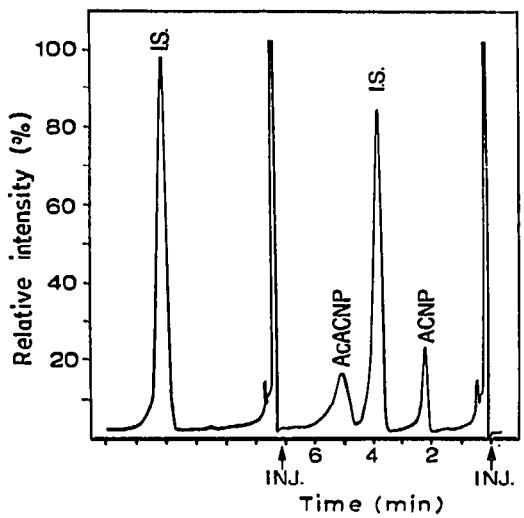


Fig. 5. Gas chromatogram of the first extract fractions from a plasma sample from a patient not receiving clonazepam, blank with added internal standard (left), and from a plasma sample from a patient receiving clonazepam (right). The concentrations of clonazepam were 0 and 45 ng/ml, respectively. I.S. = Internal standard (Ro 5-4435); CNP = clonazepam.

considered such alterations unlikely to produce sufficient improvements in separation.

A few plasma samples contained impurities that interfered in the measurement of the amino metabolite. These impurities could be removed by washing the hydro-



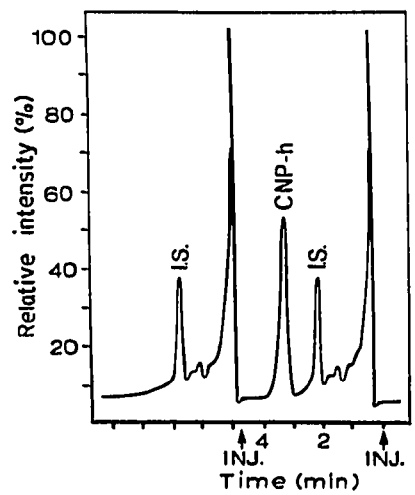


Fig. 6. Gas chromatogram of the second extract fractions from a plasma sample from a patient not receiving clonazepam, blank with added internal standard (left), and from a plasma sample from a patient receiving clonazepam (right). The concentrations of the amino metabolite were 0 and 30 ng/ml, respectively, and the concentrations of the acetamido metabolite were 0 and 9 ng/ml, respectively. ACNP = 7-Amino metabolite of clonazepam; AcACNP = 7-acetamido metabolite of clonazepam; I.S. = internal standard (acetylated Ro 10-3384).

Fig. 7. Gas chromatogram of hydrolyzates of the first extract fractions from a plasma sample from a patient not receiving clonazepam, blank with added internal standard (left), and from a plasma sample from a patient receiving clonazepam (right). The concentrations of clonazepam were 0 and 66 ng/ml, respectively. I.S. = Internal standard; CNP-h = clonazepam hydrolyzate.

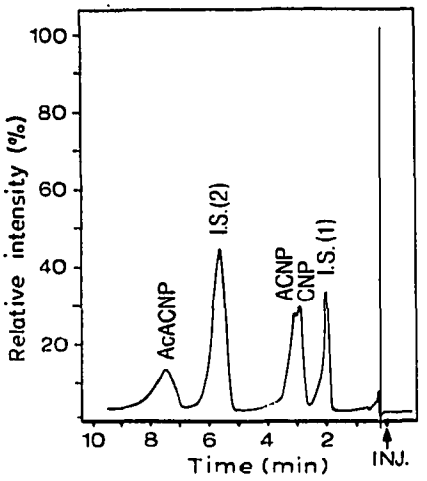


Fig. 8. Gas chromatogram of a standard solution containing clonazepam (CNP), the amino metabolite (ACNP), the acetamido metabolite (AcACNP) and the two internal standards Ro 5-4435 (I.S. (1)) and acetylated Ro 10-3384 (I.S. (2)). Column material, 1 % OV-17 on Celite J. J. CQ, 100–120 mesh. Column temperature, 270°.

chloric acid phase with toluene–ethyl acetate (1:1) before neutralization of the acid and extraction of the metabolites. This washing resulted in a loss of the acetamido metabolite. In these samples, the acetamido metabolite could only be determined with lower accuracy.

Determination of clonazepam as its benzophenone derivative involves more work than the method based on the determination of unchanged clonazepam. To compensate for this, the sensitivity is slightly higher, and the column conditions are not so critical as the benzophenones are more suitable than clonazepam for GC. The precision is at same level, but the accuracy might be lower (see below). Further, there is no possibility of determining the amino and the acetamido metabolites after hydrolysis, because the benzophenones obtained are unstable.

Specificity

Extracts containing clonazepam and its amino metabolite from patients treated with clonazepam were studied in the gas chromatograph–mass spectrometer. Fractions that had the retention times of these compounds always gave fragments characteristic of these compounds, but they also gave other fragments. These fragments were probably derived from material that was not sensed by an ECD, as they were also present in extracts from blank plasma but were not detected in the GC recordings. There was too little of the acetamido metabolite in plasma to give a usable mass spectrum.

The mass fragmentographic determination of clonazepam as its benzophenone derivative was possible (Fig. 9), but therapeutic concentrations of clonazepam were near to the sensitivity limit of the method. Only if at least 10 ml of plasma were used was the accuracy acceptable.

Mass fragmentography of clonazepam and its metabolites as unchanged com-

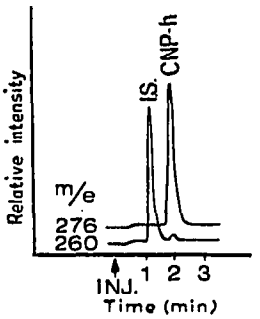


Fig. 9. Mass fragmentographic recording of the hydrolytic products (the corresponding benzophenones) of clonazepam (CNP-h) and of the internal standard (Ro 5-4435-h). Ionization energy, 30 eV. Trap current, 60 μA. Column temperature, 240°. Channel settings, *m/e* 260 (M^+ for Ro 5-4435-h) and 276 (M^+ for CNP-h).

pounds was also tried. However, insufficient sensitivity and liability to tailing of the peaks implied that only toxic concentrations could be measured by this method.

High concentrations of clonazepam in plasma samples determined by GC and by mass fragmentography were in good agreement.

GC determinations of clonazepam in plasma samples as the unchanged compound and as the benzophenone derivative showed approximately the same levels. However, the concentrations found by the method measuring the benzophenone derivative seemed on an average to be slightly higher (0–15%). The higher concentrations might be due to co-determination of some 3-hydroxyclonazepam, which would give the same benzophenone derivative as that obtained from clonazepam on acid hydrolysis (see Fig. 3).

In gas chromatograms of the first extract (not hydrolyzed) of plasma samples from patients treated with clonazepam over a long period, a small peak with the same retention time as 3-hydroxyclonazepam was sometimes present. This peak was not observed in the chromatograms of plasma blanks.

Quantitative determination

Standard curves were prepared from chromatograms of extracts of plasma to which known amounts of clonazepam and the metabolites had been added. The ratio of the peak height of the compound determined to that of the corresponding internal standard was plotted against the concentration (Fig. 10). A linear relationship was obtained.

The limit of sensitivity was found to be about 3 ng/ml for clonazepam and the acetamido metabolite and about 5 ng/ml for the amino metabolite. The lower limit varied slightly, depending on the amount of interfering material present in the plasma. The relative standard deviation for determination of clonazepam and its metabolites was calculated from analysis of twenty samples with the same plasma concentration and found to be 4% for clonazepam at the therapeutic level, and about 10% for the metabolites (probably because the second internal standard went through only some of the steps of the analysis).

The plasma levels in 25 patients undergoing continuous treatment with 6 mg

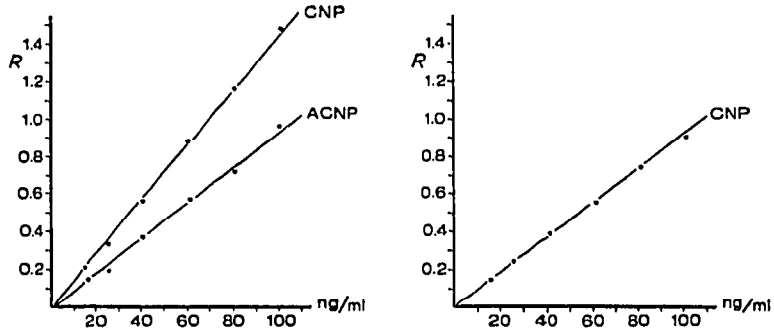


Fig. 10. Standard curves for clonazepam (CNP) and the amino metabolite of clonazepam (ACNP). Measured as unchanged compounds (left), and measured as hydrolyzates (right). R = The ratio between peak heights of CNP, ACNP or CNP-hydrolyzate and of the corresponding internal standard.

of clonazepam daily for 15–26 days were found to be 29–75 ng/ml for clonazepam, 23–137 ng/ml for the amino metabolite and <3–13 ng/ml for the acetamido metabolite.

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