Validated LC-MS/MS method for the simultaneous determination of amlodipine and its major metabolites in human plasma of hypertensive patients.

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The authors declare no conflict of interest.

Running head: Determination of amlodipine metabolites in plasma
Abstract

Background: No information on the pharmacokinetic characteristics of amlodipine (AML) metabolites is available. This study aimed to develop a method based on isocratic liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) for the simultaneous determination of AML and its two major metabolites, dehydroamlodipine (DH-AML) and O-des[2-aminoethyl]-O-carboxymethyl DH-AML (CM-DH-AML), and to use it for monitoring this drug in hypertensive patients.

Methods: Acetonitrile-deproteinized plasma specimens were separated using an octadecyl-silica column (3 µm particle size) with a mobile phase consisting of 50% methanol containing 0.15% of formic acid in water. The run time was 9 minutes. The mass spectrometer was run in the positive ion electrospray ionization mode. This method was applied for the determination of AML and its metabolites in plasma samples from patients treated with this drug.

Results: The calibration curves in human plasma of AML, DH-AML, and CM-DH-AML were linear over the concentration ranges of 0.5–64, 1–64, and 0.5–64 ng/mL, respectively, and their lower limits of quantification were 0.5, 1, and 0.5 ng/mL, respectively. Their extraction recovery rates and matrix factors in human plasma were 94.8–109.0% and 97.0–101.4%, respectively. The intra-assay and inter-assay imprecisions and accuracies were within 10.8% and 95.4–111.2%, respectively.
respectively. The plasma concentration ranges of AML, DH-AML, and CM-DH-AML were 6.5–20.9, 1.4–10.9, and 5.6–38.3 ng/mL, respectively.

**Conclusions:** The present method with acceptable analytical performance can be helpful for monitoring the plasma concentration of AML, including the determination of its metabolites in patients with hypertension.

**Key words:** amlodipine; metabolite; LC-MS/MS; human plasma; pharmacokinetics
INTRODUCTION

Amlodipine (AML) is a dihydropyridine calcium channel blocker, which is used for the treatment of hypertension and angina pectoris.\textsuperscript{1,2} AML has a long elimination half-life, which permits once-daily administration.\textsuperscript{1,3} A large number of patients are on long-term AML as a chronic treatment for hypertension. Although AML has a strong anti-hypertensive effect and shows marked clinical usefulness, adverse effects such as flushing, dizziness, headache, palpitations, and edema are occasionally observed.\textsuperscript{4,5} However, there are also cases in which the dose needs to be increased because of the poor anti-hypertensive effect.\textsuperscript{6} Inter-individual variability in the hypotensive effect of AML has been observed in clinical settings.

Like with other dihydropyridine calcium channel blockers, cytochrome P450 (CYP) 3A4 plays an important role in the metabolism of AML.\textsuperscript{7} AML is eliminated by hepatic metabolism via dehydrogenation of its dihydropyridine moiety to dehydroamlodipine (DH-AML), a pyridine derivative, and is excreted in urine largely as an oxidized pyrimidine analog, mostly as \textit{O}-des[2-aminoethyl]-\textit{O}-carboxymethyl DH-AML (CM-DH-AML) (Fig. 1).\textsuperscript{8} Some studies have examined the pharmacokinetics of AML; however, no information on the pharmacokinetic characteristics of its metabolites is available. Even for AML, the association between its pharmacokinetics and pharmacodynamics has
Some analyses using chromatographic techniques have been reported for the determination of AML in human plasma. Gas chromatography\textsuperscript{9,10} or liquid chromatography (LC) coupled to ultraviolet detection\textsuperscript{11,12} is convenient in clinical settings; however, the sensitivity and specificity of the methods are insufficient. LC coupled to tandem mass spectrometry (MS/MS) shows sensitive and specific determination, but complicated sample preparation procedures are needed in order to overcome mobile phase limitations due to the matrix effect of human plasma samples.\textsuperscript{13–18} To the best of our knowledge, no study has investigated the simultaneous determination of AML and its metabolites using human plasma in clinical settings. The difference in polarities between the parent drug and its metabolites requires optimization of the pretreatment process and LC conditions. Furthermore, the presence of chlorine atoms, of which there are two principal stable isotopes $^{35}\text{Cl}$ and $^{37}\text{Cl}$, in molecules of AML (monoisotopic mass, 408.15) and DH-AML (406.15) necessitates sufficient separation of analytes using an LC system.

To be able to determine the metabolites of AML as well as their parent drug would be useful for evaluating the pharmacokinetics of AML in patients with hypertension and elucidating inter-individual variations in the antihypertensive effect of AML observed in clinical settings. The aim of this study
was to develop a validated LC-MS/MS method for simultaneous determination of AML and its major metabolites in human plasma and to use it for monitoring of the drug in hypertensive patients.

MATERIALS AND METHODS

Chemicals
AML besylate, DH-AML oxalate, and \textit{d4}-AML as an internal standard (IS) were obtained from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). CM-DH-AML was purchased from Alsachim (Illkirch Graffenstaden, France). HPLC-grade acetonitrile and methanol, and ammonium acetate were obtained from Wako Pure Chemicals (Osaka, Japan).

Solutions
Stock solutions of the analytes were prepared at a concentration of 1 µg/mL in methanol. A stock solution (1 µg/mL) of the IS was prepared in acetonitrile. All solutions were stored at 4°C. Standard solutions of the chemicals were obtained by the dilution of stock solution with methanol for analytes and with acetonitrile for IS. Calibration standards were prepared in drug-free human plasma (Kohjin-Bio Co. Ltd., Sakado, Japan). The final plasma concentrations of AML and CM-
DH-AML were 0.5, 1, 2, 4, 8, 16, 32, and 64 ng/mL and of DH-AML were 1, 2, 4, 8, 16, 32, and 64 ng/mL, respectively. Quality control (QC) and lower limits of quantification (LLOQ) samples contained AML and CM-DH-AML concentrations of 0.5, 1, 8, and 32 ng/mL and DH-AML concentrations of 1, 2, 8, and 32 ng/mL in drug-free plasma.

**Sample pretreatment**

Ethylenediaminetetraacetic acid (EDTA)-treated plasma was obtained by centrifugation of the blood specimens at 1670 × g at 4°C for 10 minutes. Acetonitrile (600 µL) and IS (50 ng/mL, 100 µL) were added to 200 µL of the plasma samples. The mixture was ultrasonicated for 15 minutes. The samples were centrifuged at 13,000 × g at 4°C for 10 minutes and then a 750-µL aliquot of the supernatant was evaporated to dryness by rotary vacuum evaporation without heating. The residue was reconstituted with 120 µL of mobile phase and then filtrated with a syringe-driven filter unit (Millex®-LH, 0.45 µm, 4 mm, Merck Millipore, Bedford, MA, USA) before injecting into the LC system.

**Chromatographic conditions**

The analytes were separated using a UFLC\textsubscript{XR} system (Shimadzu Corporation, Kyoto, Japan). The LC system consisted of CBM-20A, DGU-20A\textsubscript{5R}, LC-
20AD\textsubscript{XR}, SIL-20AC\textsubscript{XR}, and CTO-20AC. Separation of AML and its metabolites in plasma specimens was performed with an octadecyl-silica (ODS) column (3-\textmu m particle size; TSK-gel\textsuperscript{\textregistered} ODS-100Z, 75 × 2.0 mm i.d., Tosoh, Tokyo, Japan). The mobile phase was 50% methanol containing 0.15% formic acid in water (v/v). The flow rate was 0.25 mL/minute, and the column temperature was set at 40°C. The injection volume of the autoinjector was fixed at 10 \mu L under a 4°C cooling system.

**Mass spectroscopic conditions**

The column effluent was monitored using a triple quadrupole mass spectrometer (3200 QTRAP\textsuperscript{\textregistered}, AB Sciex, Foster City, CA, USA) equipped with an electrospray probe in positive ionization mode (Analyst software version 1.6.1.). The ion transitions were monitored using a dwell time of 250 milliseconds for each compound: AML, \textit{m/z} 409.2/238.2; DH-AML, \textit{m/z} 408.2/259.3; CM-DH-AML, \textit{m/z} 423.3/287.2; and IS, \textit{m/z} 413.2/238.2. Samples were introduced to the interface through a turbo ionspray with the temperature set at 600°C. A high positive voltage of 5.5 kV was applied to the ion spray. Collision gas, curtain gas, ion source gas 1, and ion source gas 2 were set at 3, 25, 70, and 60 psi, respectively. Collision energies for AML, DH-AML, CM-DH-AML, and IS were −15, −35, −35, and −15 volts, respectively.
Selectivity, calibration curves, and sensitivity

Selectivity of the method was evaluated by analyzing six independent drug-free plasma samples. Linear regression analysis of AML and its metabolites was performed by plotting the peak area ratio of the analyte to IS versus analyte concentration. The linearity of AML, DH-AML, and CM-DH-AML was observed at plasma concentration ranges of 0.5–64, 1–64, and 0.5–64 ng/mL, respectively. LLOQs were defined as the concentration of analyte with an accuracy within 80–120% and an imprecision less than 20%.

Analytical performance

Pretreatment recoveries were assessed by comparing the mean peak areas of the regularly prepared QC samples extracted from spiked matrix with the mean peak areas of standard samples spiked post extraction. Matrix effects on ionization were assessed as matrix factors by comparing the mean peak areas of standard samples spiked post extraction with the mean peak areas of standard samples in the mobile phase. Accuracy (expressed as % error) and imprecision (expressed as % relative standard deviation, RSD) were evaluated with three QC samples. Intra-assay and inter-assay imprecision and accuracy were determined by analyzing five sets of samples.
Stability of AML and its metabolites

The stabilities of AML, DH-AML, and CM-DH-AML in human plasma were evaluated by comparing peak areas after 24 hours of storage at 4°C and room temperature with initial peak area. Long-term stabilities in plasma at −80°C were determined after one month. The effect of freeze and thaw cycles on the stability of plasma samples containing the analytes was determined by subjecting three QC samples to two freeze-thaw cycles. The stability test of stock solutions stored for a month at 4°C was determined. In order to estimate the stability of the analytes stored in the prepared sample, QC samples were kept in an autoinjector maintained at 4°C for 24 hours.

Clinical application

The present study was performed in accordance with the Declaration of Helsinki and its amendments. The Ethics Committee of Hamamatsu University School of Medicine approved the protocol. A total of 12 Japanese patients treated with oral AML besylate for essential hypertension at Hamamatsu University Hospital were enrolled. All patients received 5 mg of oral AML once daily for at least 7 days. The exclusion criteria were patients with non-compliance or concomitant use of a strong CYP3A modifier such as rifampicin or triazole antifungal agents.
Blood specimens from a forearm vein were drawn into tubes containing EDTA dipotassium 24 hours after the AML treatment. Plasma concentrations of AML and its metabolites were evaluated as the absolute plasma concentration and its dose and body weight-adjusted values. Metabolism of AML was estimated by the plasma concentration ratio of the metabolites to AML as metabolic ratio.

RESULTS

Mobile phase and chromatography

The analytes were protonated in a mobile phase of 50% methanol containing 0.15% of formic acid in water. Figure 2 shows the MS/MS chromatograms of AML and its two metabolites in human plasma. AML, DH-AML, CM-DH-AML, and IS were eluted at 4.1, 1.9, 7.0, and 4.1 minutes, respectively, with a total run time of 9 minutes. One peak of IS was observed in the chromatograms of a drug-free plasma sample (Figure 2A) and a plasma sample from a hypertensive patient not receiving amlodipine (Figure 2B).

Selectivity, calibration curves, and sensitivity

AML, DH-AML, CM-DH-AML, and IS were separated and no endogenous interference from 6 different batches of human plasma was observed (Fig. 2).
The calibration curves in human plasma of AML, DH-AML, and CM-DH-AML were linear \((r > 0.999)\) over the concentration ranges of 0.5–64, 1–64, and 0.5–64 ng/mL, respectively. The LLOQs of AML, DH-AML, and CM-DH-AML were 0.5, 1, and 0.5 ng/mL, respectively.

**Analytical performance**

The pretreatment recovery rates of AML, DH-AML, and CM-DH-AML were 109.0 ± 5.1%, 94.8 ± 7.9%, and 99.5 ± 4.6%, respectively. The matrix factors were 99.0 ± 2.4% for AML, 101.4 ± 8.8% for DH-AML, and 97.0 ± 10.0% for CM-DH-AML. Table 1 shows the analytical performance of the present method. The intra- and inter-assay accuracies of AML, DH-AML, and CM-DH-AML were 97.5–111.2% and 97.0–103.5%, 97.9–109.0% and 98.1–103.0%, and 99.4–111.0%, and 95.4–102.8%, respectively. The intra- and inter-assay imprecision of AML, DH-AML, and CM-DH-AML were 2.7–3.9% and 1.0–8.8%, 1.9–7.7% and 5.0–8.8%, and 3.5–9.0% and 3.5–10.8%, respectively.

**Stability**

AML, DH-AML, and CM-DH-AML remained at 99.6%, 107.7%, and 93.1% of the initial values at 4°C and 97.8%, 107.5%, and 91.1% at room temperature after 24 hours of storage in human plasma, respectively. All analytes remained at
acceptable levels after two cycles of freeze and thaw. AML, DH-AML, and CM-DH-AML were stable in plasma specimens stored at −80°C for at least one month. The stock solutions of AML, DH-AML, and CM-DH-AML were stable for three months. The prepared samples of AML, DH-AML, and CM-DH-AML were stable in the autoinjector that was maintained at 4°C for 24 hours.

**Plasma concentrations of AML and its metabolites in patients**

Figure 3 shows the plasma concentrations of AML, DH-AML, and CM-DH-AML in 12 hypertensive patients presented in Table 2. The plasma concentration ranges were 6.5–20.9, 1.4–10.9, and 5.6–38.3 ng/mL, respectively. The plasma concentrations of AML, DH-AML, and CM-DH-AML in each patient were within the ranges of the calibration curves. The median and interquartile range (IQR) of the plasma concentrations of AML, DH-AML, and CM-DH-AML were 11.6 and 8.93–17.0, 3.4 and 2.51–4.21, and 11.7 and 9.33–18.3 ng/mL, respectively.

**Variation in plasma exposure and metabolic ratio**

Table 2 shows the characteristics of the patients enrolled in this study. The median and IQR of the dose and weight-adjusted plasma concentrations for AML, DH-AML, and CM-DH-AML were 138 and 113–155, 31 and 26–42, and
126 and 102–208 ng/mL per mg/kg in patients with hypertension, respectively. The median metabolic ratios of DH-AML and CM-DH-AML to AML were 0.27 (IQR, 0.20–0.47) and 1.2 (0.80–1.6), respectively.

**DISCUSSION**

To the best of our knowledge, this is the first report on a validated LC-MS/MS method for the simultaneous determination of AML metabolites together with the parent drug in human plasma. This report presents the plasma concentration ranges of two major metabolites of AML in hypertensive patients. In the pretreatment process, the present method requires only acetonitrile, a readily available organic solvent that has a wide range of clinical applications. The analytical run time of this method was within 10 minutes, rapid enough for the determination of plenty of samples in succession. In addition, the present method has acceptable analytical performance according to international guidance.

The method can be applied to evaluating the pharmacokinetics of AML including the determination of its metabolites in clinical settings.

This study adopted a simple deproteinizing method using acetonitrile, and the mean values of the pretreatment recovery rates of AML, DH-AML, and CM-DH-AML were around 100%. Massaroti et al. employed liquid–liquid
phase extraction using a mixture of ethyl acetate and hexane with higher toxicity. Bhatt et al. used solid-phase extraction, and its recovery rate for AML was approximately 60%. Additively, these methods determine only AML, while our present method measured three different analytes possessing different polarities concomitantly with adequate recovery. In this method, 200 µL of human plasma was sufficient for sample pretreatment, while the reported methods of Zou et al. and Bhatt et al. required 1000 µL of plasma specimen. 

Our method takes 9 minutes to determine AML, DH-AML, and CM-DH-AML. Carvalho et al. reported a method taking 5 minutes, and the method of Nirogi et al. ran 1.5 minutes though they measured only AML. Better separation using an LC system was needed in the simultaneous determination of AML and DH-AML because of the chlorine atom contained in the molecules of these compounds. Basically the use of stable isotopes of chlorine ($^{35}$Cl and $^{37}$Cl) could cause problems in the detection of AML and DH-AML using a mass spectrometer. The reason is that the difference in the value of the molecular weight between AML and DH-AML is 2, due to the abstraction of two hydrogen atoms in the dehydrogenation process from AML to DH-AML. However, our method employed a high carbon-content ODS column (20% with 3 µm particle size) to conquer this problem and delivered sufficient separation of AML, DH-AML, and CM-DH-AML with isocratic elution.
The LLOQ of AML in the present method was 0.5 ng/mL. Yasuda et al. described a method to determine AML with an LLOQ of 0.014 ng/mL. However, the minimum plasma concentration of AML in hypertensive patients was 6.5 ng/mL in the present study, so our method has enough sensitivity for its application to clinical settings. This method has accuracies within the range of 85–115% and imprecisions within 15%. These results met the criteria of international guidance for bioanalytical method validation. The three analytes were stable in human plasma at room temperature for 24 hours and were still present at acceptable levels after two cycles of freeze and thaw. These results indicate that there was no deterioration of samples from blood collection until sample preparation with no need for unconventional storage conditions. The prepared samples were stable in the autoinjector for 24 hours. Thus, the present method enables the continuous determination of a large number of samples.

In this study, the method was applied to determine the plasma concentrations of AML, DH-AML, and CM-DH-AML in 12 hypertensive patients. The plasma concentrations of three analytes in each patient were within the ranges of the calibration curves. The median of the plasma concentration of AML was 11.6 ng/mL in this study. De Nicolo et al. reported the plasma AML concentration of 12 hypertensive patients, and its median was 10.9 ng/mL. The mean value of the plasma AML concentration reported by Faulkner et al. was
11.8 ng/mL in 28 healthy subjects receiving repeated oral administration. The plasma AML concentration in patients in the present study was similar to those in earlier reports. In addition, this study showed the plasma concentration ranges of DH-AML and CM-DH-AML in clinical settings for the first time.

We employed the present method to determine only pre-dose concentration of AML and its metabolites in this study. This method could be applicable to the evaluation of not only pre-dose but also post-dose concentrations of AML and its metabolites. The range of our calibration curve for AML covers the reported peak concentration after a single oral administration of 10 mg AML (5.9 ng/mL) and after 14 repeated oral administrations of 15 mg AML (18.1 ng/mL). As for the metabolite concentrations, no data in clinical settings are available. In our study, the medians of the plasma concentration and its ranges of calibration curves of DH-AML and CM-DH-AML were 3.4 and 1–64, and 11.7 and 0.5–64 ng/mL, respectively. The plasma concentrations of the metabolites congregated in lower region of their calibration curves, so we consider that the calibration curves of the metabolites could also cover the peak concentrations. However, the lower plasma concentrations of DH-AML have a large inter-individual variation. There is a concern that DH-AML concentration might be undetectable after a single oral administration.
Large individual variations were observed in the plasma concentrations of AML, DH-AML, and CM-DH-AML after adjusting for body weight and dose. The metabolic ratios of DH-AML and CM-DH-AML also showed a large inter-individual variation. AML is metabolized by hepatic CYP3A4, the activity of which shows large individual differences based on genetic or environmental factors. CYP3A4 activity is potentially responsible for the inter-individual variabilities in plasma concentrations of AML and its metabolites, though further investigation is needed to identify the factors that affect AML pharmacokinetics.

The present method has a few limitations. First, this method was applied to population with the present patient characteristics in Table 2. Body weight, estimated glomerular filtration rate, and total bilirubin ranged from 32.5–72.8 kg, 6–88 mL/minute/1.73 m², and 0.2–1.0 mg/dL, respectively. Further verification of the ranges of calibration curves is required, especially in patients with severe hepatic failure or severe renal disorder since AML is metabolized in the liver and then excreted in the kidney. These detailed characteristic data of enrolled patients in Table 2 may be useful for the evaluation of suitability of the present method to other special populations. Second, we evaluated only pre-dose plasma concentrations of AML and its metabolites in this study. The determined concentration of AML could be considered as a pre-dose concentration at steady state because all enrolled patients received AML at least 7 days, which is enough
time to reach steady state estimated by the half-life of AML. As for the metabolites, further investigation is needed to determine the plasma concentration profile, as so far only few data on the pharmacokinetic characteristics of the AML metabolites are available. Third, the method was used only in essential hypertension patients. Early postpartum women had higher concentrations of AML, though the reported concentration was within the range of our calibration curve.\textsuperscript{23} The reported AML concentration in patients with stable angina pectoris was also within the measurable range of the present method.\textsuperscript{24} For these indications, our method with its large calibration ranges of AML and its metabolites could be applied in clinical settings.

One of the clinical implications of the present method is to clarify the pharmacokinetic characteristics of the AML metabolites in clinical settings. Moreover, the metabolic ratio of AML determined by this method could be applied to monitor CYP3A4 activity. In general, the metabolic ratio of midazolam is used as a biomarker of hepatic CYP3A activity, for example, to detect the induction or genetic variants of drug-metabolizing enzymes in healthy-subject study. However, midazolam as a probe drug cannot be administered to the elderly and pregnant women. AML is specifically metabolized by CYP3A4\textsuperscript{7} and commonly prescribed for various populations including the elderly and women with pregnancy-induced hypertension. Thus,
our present method is expected to be useful for clinical investigations as a biomarker of CYP3A4 activity, as well as pharmacokinetic studies of AML.

CONCLUSIONS
This study presents a validated LC-MS/MS method for the simultaneous determination of AML and its two major metabolites in human plasma. The present method with an acceptable analytical performance can be helpful for monitoring the pharmacokinetics of AML, including the determination of its metabolites in patients with hypertension and elucidating inter-individual variations in the hypotensive effect of AML observed in clinical settings.
REFERENCES


Figure legends

**FIGURE 1.** Chemical structures of amlodipine and its metabolites: (A) amlodipine, (B) dehydroamlodipine, and (C) O-des[2-aminoethyl]-O-carboxymethyl dehydroamlodipine.

**FIGURE 2.** MS/MS chromatograms of amlodipine and its metabolites in human plasma: (A) a drug-free plasma sample, (B) a plasma sample from a hypertensive patient not receiving amlodipine, (C) a plasma sample spiked with amlodipine: 16 ng/mL; dehydroamlodipine: 32 ng/mL; and O-des[2-aminoethyl]-O-carboxymethyl dehydroamlodipine: 16 ng/mL, and (D) a plasma sample from a patient receiving 5 mg amlodipine: retention times for (1) amlodipine, (2) dehydroamlodipine, (3) O-des[2-aminoethyl]-O-carboxymethyl dehydroamlodipine, and (4) internal standard (IS) are 4.1, 1.9, 7.0, and 4.1 minutes, respectively. One peak of IS was observed in the chromatograms of a drug-free plasma sample (A) and a plasma sample from a hypertensive patient not receiving amlodipine (B).

**FIGURE 3.** Plasma concentrations of amlodipine (AML), dehydroamlodipine (DH-AML), and O-des[2-aminoethyl]-O-carboxymethyl dehydroamlodipine (CM-DH-AML) in hypertensive patients (n = 12).
Table 1  Intra- and inter-assay imprecisions and accuracies of AML and its metabolites in human plasma

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<th>Analytes</th>
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<td></td>
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<td>Mean ± SD (ng/mL)</td>
<td>Accuracy (%)</td>
<td>RSD (%)</td>
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<td></td>
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<td></td>
<td>1.11 ± 0.04</td>
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<td>CM-DH-AML</td>
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<td>99.4</td>
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AML, amlodipine; DH-AML, dehydroamlodipine; CM-DH-AML, O-des[2-aminoethyl]-O-carboxymethyl DH-AML; SD, standard deviation; and RSD, relative standard deviation
Table 2  Patient characteristics

<table>
<thead>
<tr>
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<td>Age, years</td>
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<td>Male/Female</td>
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<td>Body weight, kg</td>
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<td>Total protein, g/dL</td>
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<td>Serum albumin, g/dL</td>
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<td>Serum creatinine, mg/dL</td>
<td>0.92 (0.49–7.83)</td>
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<tr>
<td>Blood urea nitrogen, mg/dL</td>
<td>19.4 (9.4–79.1)</td>
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<td>Estimated glomerular filtration rate, mL/min/1.73 m²</td>
<td>51 (6–88)</td>
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<tr>
<td>Aspartate aminotransferase, IU/L</td>
<td>19 (12–38)</td>
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<td>Alanine aminotransferase, IU/L</td>
<td>17 (5–51)</td>
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<tr>
<td>Total bilirubin, mg/dL</td>
<td>0.6 (0.2–1.0)</td>
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</table>

Data are represented as median and range in parentheses.
(A) \[ \text{Dehydrogenation} \]

\[
\begin{align*}
\text{(A)} & \quad \text{Cl} & \quad \text{O} & \quad \text{O} & \quad \text{NH}_2 \\
\text{(B)} & \quad \text{Cl} & \quad \text{O} & \quad \text{O} & \quad \text{O} & \quad \text{NH}_2 \\
\text{(C)} & \quad \text{Cl} & \quad \text{O} & \quad \text{O} & \quad \text{O} & \quad \text{COOH}
\end{align*}
\]