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SIMULTANEOUS DETERMINATION OF AMINOPYRINE AND ITS METABOLITES IN RAT PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple and sensitive high-performance liquid chromatographic method has been developed for the quantitative determination of aminopyrine and eight of its metabolites (4-methylaminoantipyrine, 4-aminoantipyrine, 4-acetylaminoantipyrine, 4-acetylmethylaminoantipyrine, 4-hydroxyantipyrine, 4-formylaminoantipyrine, 3-hydroxymethyl-2-methyl-4-dimethylamino-1-phenyl-3-pyrazolin-5-one and 2-methyl-4-dimethylamino-5-oxo-1-phenyl-3-pyrazoline-3-carboxylic acid) in plasma of rats The procedure includes a chloroform extraction prior to chromatography and quantitative analysis using peak-area ratios (ultraviolet absorbance detection at 260 nm) of aminopyrine and its metabolites to the internal standard, phenobarbital The concentration—response curves for these compounds were linear from 20 ng/ml to 200 μg/ml The extraction coefficients of aminopyrine and its metabolites from plasma were 80–100% The coefficients of variation of intra- and inter-assay for these compounds in plasma were less than 2 5—5 5% This method has been found to be applicable to the analysis of plasma samples obtained from aminopyrine-dosed rats

INTRODUCTION

There are many reports on the metabolites of aminopyrine (AM) in humans and in experimental animals Previously, it was reported that 4-methylamino-antipyrine (MAA), 4-aminoantipyrine (AA), 4-formylaminoantipyrine (FAA) and 4-acetylaminoantipyrine (AcAA) were metabolized from AM in plasma of humans and animals [1, 2] Although the AM metabolites 4-hydroxyantipyrine (AMOH), 4-acetylmethylaminoantipyrine (AcMAA), 3-hydroxymethyl-2-methyl-4-dimethylamino-1-phenyl-3-pyrazolin-5-one ($AMCH_2OH$) and 2-methyl-4-dimethylamino-5-oxo-1-phenyl-3-pyrazoline-3-carboxylic acid

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(AMCOOH) were identified in the urine and in the hepatocyte system isolated from the rat and the rabbit [3–6], they were not identified in the plasma Therefore, we decided to investigate some metabolites of AM in the plasma of rats

Several methods have been developed for the simultaneous determination of AM and its metabolites in biological fluids. Some of these methods, based on colorimetric, spectrophotometric [3] and thin-layer chromatographic [7–9] procedures, are only semi-quantitative with a low sensitivity. Gas chromatographic methods using hydrogen flame ionization detection [10], and the gas chromatographic—mass spectrometric method [11–13] have a higher sensitivity and selectivity, but they involve extensive time-consuming purification and derivatization steps. Recently, a high-performance liquid chromatographic (HPLC) method [14–17] has been reported Inoue et al. [16] used a reversed-phase high-performance liquid chromatographic (RP-HPLC) method to determine AM metabolites (AMOH, MAA, AA and FAA) in the liver microsome.

In this paper, we reported a simple and rapid method using HPLC for the simultaneous determination of AM and eight metabolites (AMCOOH, FAA, AcAA, $AMCH_2OH$, AcMAA, MAA, AA and AMOH) in rat plasma This method is more sensitive and selective than other methods reported in the literature, and is suitable for the routine determination of AM and its metabolites in rat plasma

EXPERIMENTAL

Reagents

JP-X grade AM (Hoei Yakko), phenobarbital (Fujinaga Seiyaku) and reagent grade AA (Nakarai Chemical, Kyoto, Japan) were used Acetonitrile (special grade for liquid chromatography) was obtained from Wako Pure Chemical Industries. MAA, AcAA, AcMAA, AMOH, $AMCH_2OH$, FAA and AMCOOH were prepared by the known method [18–23] All other chemicals were of analytical-reagent grade

Animal experiments

Male Wistar rats weighing 180–200 g were used The rats were kept on a commercial diet (Oriental Yeast) and were fasted for 20 h prior to AM administration. Water was given freely AM was orally or intravenously administered to the rats A 1 0-ml aliquot of an aqueous solution of AM (100 mg/kg) was orally administered to the rat and 1 ml of saline solution containing 35 mg/kg AM was injected into the femoral vein

Equipment

The HPLC apparatus consisted of a Hitachi Model 655 solvent delivery system, a Hitachi Model 635 M absorbance detector monitoring at 260 nm and a Hitachi Model 833 chromatoprocessor Separations were performed on a reversed-phase column (25 cm × 4 6 mm I D) with ODS-120A (particle size 5 μm, Toyo Soda, Japan) Aliquots of 20 μl were injected into the chromatograph All separations were done at ambient temperature using methanol—0 05 *M* phosphate buffer, pH 3 9 (30 70, v/v) as a mobile phase at

the flow-rate of 0.7 ml/min, producing a pressure of 1400 MPa. The solvent was subjected to ultrasonic waves before use.

Extraction procedure

A 0.25-ml blood sample was collected from a rat by the procedure of Weeks and Davis [24] and Ashley and Levy [25], and immediately centrifuged at 700 *g* for 30 min. Supernatant (0.1 ml) and chloroform (5 ml) were added to the centrifuge tube, the tube was shaken for 30 min and centrifuged at 1500 *g* for 10 min. A 4-ml volume of the extract was put into another centrifuge tube. Subsequently, the mixture was adjusted to pH 3.5 with 0.1 *M* hydrochloric acid (ca. 0.1 ml) and was similarly extracted with 4 ml of chloroform. A 4-ml portion of the chloroform phase was combined with the previous 4 ml of chloroform, following the same procedure. The combined extracts were evaporated to dryness. The residue was reconstituted in 0.1 ml (300 μg/ml) of the mobile phase containing the internal standard (phenobarbital) and 20-μl aliquots were injected into the HPLC system.

Standard curves

No detectable AM or its metabolites was found in the pooled plasma from rats by this method. We used deionized water and the pooled blank plasma to make standard concentration curves ranging from 20 ng/ml to 200 μg/ml AM, AA, MAA, AcAA, AcMAA, AMOH, $AMCH_2OH$, FAA and AMCOOH. The standard curves were calculated by plotting the ratio of peak area of AM (or its metabolites) and internal standard against known concentrations of these compounds contained in plasma samples. Linear regression lines were calculated by the least-squares method.

Recovery experiments

After known amounts of AM and its metabolites were added to water and to the pooled rat plasma, these compounds were analysed, and the analytical recoveries of the exogenous components were calculated.

Reproducibility

We used pooled samples of the rat plasma, supplemented with a known concentration (20 μg/ml) of AM and its metabolites. As an intra-assay reproducibility, the same samples were determined six times within a day, and for inter-assay reproducibility the same samples were determined within an additional six days. The coefficients of variation (C.V., %) were calculated from the mean values and the S.D. values.

Thin-layer chromatography

A 5-ml volume of plasma was extracted with chloroform 3 h after oral administration of AM (100 mg/kg) to the rat. The extracts obtained from at least five rats were concentrated into a small volume (75 μl), which was applied on a silica gel plate (20 × 20 cm, Kieselgel 60 F-254, Merck) by repeated spotting. The plate was developed with chloroform—acetone (8:2, v/v, solvent in the first dimension) and dichloromethane—acetone (6:4, v/v, solvent in the second dimension) using a two-dimensional arrangement. Compounds were located under UV light.

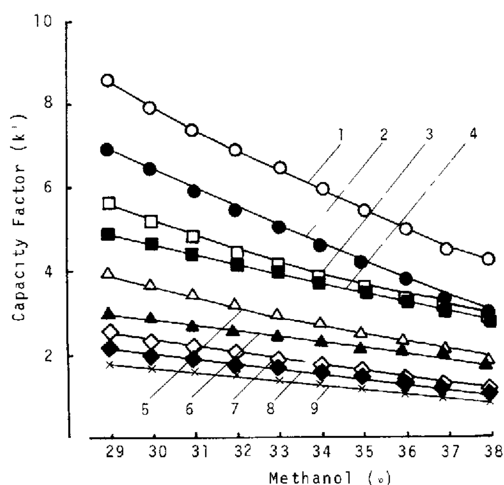
RESULTS AND DISCUSSION

The influence of methanol concentration in the mobile phase on the separation of AM and eight metabolites is shown in Fig 1 The capacity factors (k') resemble each other with increasing methanol concentration These compounds are well separated at a 30.70 ratio of methanol--0 05 *M* phosphate buffer (pH 3 9)

Fig 2 shows a typical chromatogram of AMCOOH, FAA, AcAA, $AMCH_2OH$, AcMAA, MAA, AA, AMOH, AM (10 μg/ml) and the internal standard (300 μg/ml)

Typical chromatograms of blank rat plasma extracts, plasma extracts spiked with AM and eight of its metabolites, and plasma extracted 180 min after oral administration of AM to rat are shown in Fig 3 Complete separation of the eight metabolites was obtained, with the following retention times AMCOOH, 11 2 min, FAA, 12 3 min, AcAA, 13 8 min, $AMCH_2OH$, 16 0 min, AcMAA, 19 5 min, MAA, 23 7 min, AA, 26 0 min, AMOH, 31 4 min, AM, 37 1 min, phenobarbital (internal standard), 42 1 min The lower limit of detection was 20 ng/ml When drug-free plasma was extracted in the same manner, no interference from endogenous plasma constituents was observed The assay time for HPLC was ca 50 min

Calibration curves for AM and its metabolites in plasma were linear in the concentration range 20 ng/ml to 200 μg/ml The regression equation obtained from data on standards in plasma, when peak-area ratios were taken, were for AMCOOH, y = 0 0162x + 0 0001, r = 0 9984, for FAA, y = 0 0388x + 0 0079, r = 0 9987, for AcAA, y = 0 0342x + 0 0055, r = 0 9887, for $AMCH_2OH$, y =



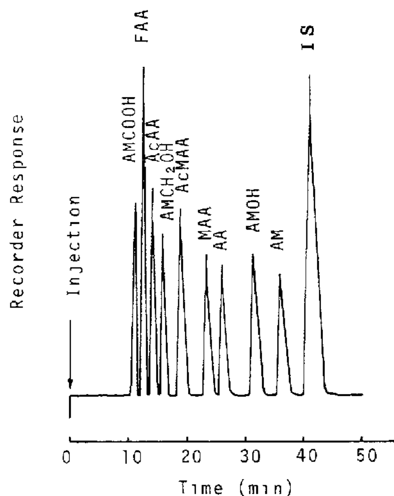


Fig 1 Relationship between capacity factor (k') and methanol concentration in the mobile phase Column prepacked column (Toyo Soda), 5 μm ODS-120A, 25 × 4 6 mm I D , mobile phase various percentages of 0 05 *M* phosphate buffer (pH 3 9)—methanol, flow-rate 0 7 ml/min 1 = AM, 2 = AMOH, 3 = AA, 4 = MAA, 5 = AcMAA, 6 = $AMCH_2OH$, 7 = AcAA, 8 = FAA, 9 = AMCOOH

Fig 2 A typical chromatogram of a mixture of aminopyrine, its metabolites (10 μg/ml each) and internal standard (I S , phenobarbital, 300 μg/ml)

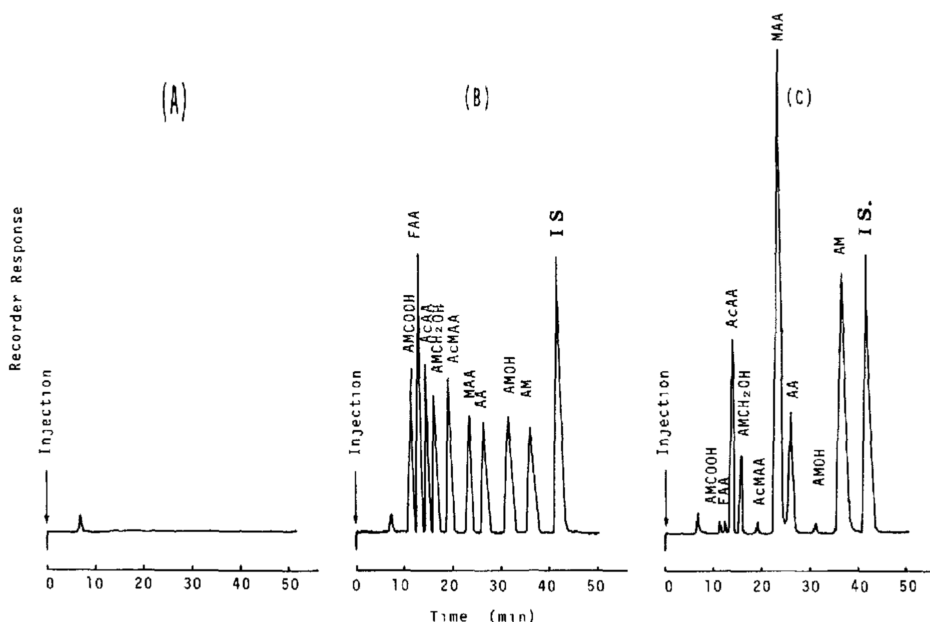


Fig 3 A typical chromatogram of plasma extracts (A) Drug-free plasma without internal standard (B) Plasma spiked with AMCOOH, FAA, AcAA, $AMCH_2OH$, AcMAA, MAA, AA, AMOH, AM (10 μg/ml each) and internal standard (phenobarbital, 300 μg/ml) (C) Plasma at 180 min after oral administration of AM (100 mg/kg) in rats

$0.0315x + 0.0053$, $r = 0\ 9979$, for AcMAA, $y = 0\ 0417x + 0\ 0009$, $r = 0.9821$; for MAA, $y = 0\ 0379x + 0\ 0038$, $r = 0\ 9983$, for AA, $y = 0.0423x + 0\ 0008$, $r = 0\ 9977$, for AMOH, $y = 0\ 0447x + 0\ 0011$, $r = 0\ 9976$, for AM, $y = 0\ 0365x + 0\ 0008$, $r = 0\ 9977$

The precision of the method was determined by repeated analysis of plasma specimens containing known concentrations (20 μg/ml) of AM and its metabolites As shown in Table I, the within-run precision was between 2.5 and 4.5%

TABLE I

PRECISION OF ASSAY OF AMINOPYRINE AND ITS METABOLITES

Concentration of AM and its metabolites added was 20 μg/ml

Drug	Concentration found (mean ± S D) (μg/ml)		Coefficient of variation (%)	
	Within-run	Day-to-day	Within-run	Day-to-day
AM	20 04 ± 0 89	20 70 ± 1 04	4 4	5 2
AMCOOH	20 08 ± 0 81	19 89 ± 0 83	4 1	4 2
FAA	20 04 ± 0 50	20 09 ± 1 00	2 5	5 0
AcAA	19 99 ± 0 69	19 99 ± 1 11	3 5	5 5
$AMCH_2OH$	20 01 ± 0 50	19 97 ± 1 00	2 6	5 2
AcMAA	19 99 ± 0 81	20 03 ± 1 11	4 1	5 5
MAA	20 05 ± 0 54	20 07 ± 0 97	2 8	4 9
AA	20 06 ± 0 81	20 00 ± 0 94	4 1	4 7
AMOH	20 00 ± 0 89	19 95 ± 1 00	4 5	5 0

TABLE II

ANALYTICAL RECOVERY STUDIES IN PLASMA

Drug	Recovery (mean ± S D , n = 12) (%)
AM	101 07 ± 4 78
AMCOOH	81 71 ± 3 53
FAA	99 09 ± 6 65
AcAA	99 61 ± 3 05
$AMCH_2OH$	98 24 ± 4 50
AcMAA	97 66 ± 5 92
MAA	100 52 ± 2 49
AA	99 40 ± 2 39
AMOH	99 42 ± 3 26

The analytical recovery of AM and its metabolites in the pooled plasma of rats is shown in Table II The recovery was determined by comparing the peak area of AM and its metabolites and the internal standard. Although the recovery of AMCOOH was 81.7%, the recovery of other compounds was between 97 and 101%.

In the preliminary observation by HPLC, the plasma extracted from five rats after oral administration of AM showed peaks that seemed to be AMCOOH, $AMCH_2OH$, AMOH and AcMAA These metabolites were detected in the urine and in the isolated hepatocyte system prepared from rats by several investigators, but there was no information on these metabolites in rat plasma. Therefore, by two-dimensional thin-layer chromatography, we analysed whether or not these metabolites existed in the rat plasma Fig 4 shows a

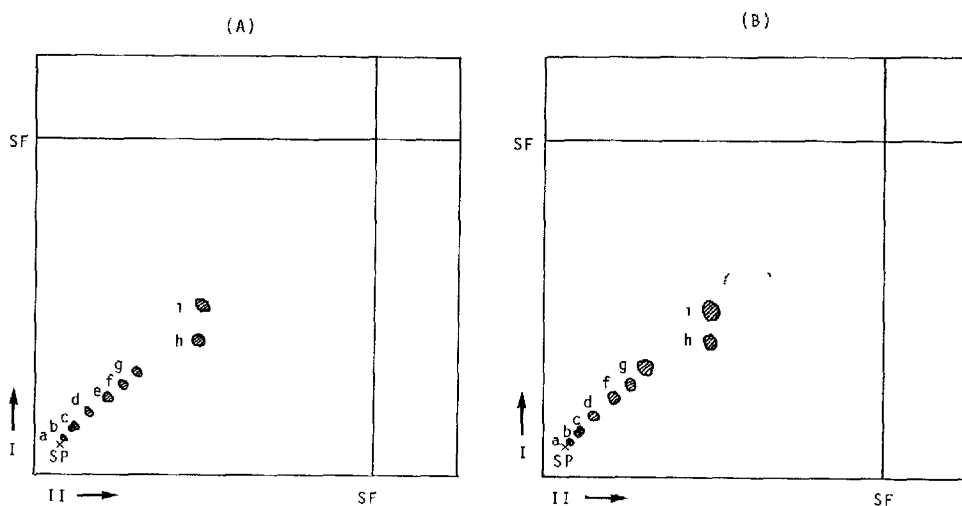


Fig 4 Two-dimensional thin-layer chromatogram of aminopyrine and its metabolites TLC was carried out in chloroform—acetone (8 2, v/v, solvent in the first dimension) and dichloromethane—acetone (6 4, v/v, solvent in the second dimension) The spots seen on the chromatogram in UV light are indicated (A) Separation of authentic samples (B) Separation of rat plasma extract after administration of AM a = AMCOOH, b = AcAA, c = FAA, d = AcMAA, e = AA, f = $AMCH_2OH$, g = MAA, h = AMOH, i = AM, SF = solvent front, SP = origin

typical chromatographic separation of AM and its metabolites The R_F value of the authentic samples completely agreed with the spots of extract from rat plasma after the administration of AM.

The plasma samples were drawn at 15, 30, 60, 120, 180, 300, 480 and 720 min after oral and intravenous administration of AM in rats. The plasma was separated and analysed as described above Results of the analysis were plotted as a concentration—time curve Fig 5 shows the plasma concentration of AM and the eight metabolites, following oral administration of AM AM was metabolized so rapidly that the two metabolites, MAA and AA, were detected even in the early stages. The plasma concentrations of other metabolites (AMCOOH, FAA, AcAA, $AMCH_2OH$, AcMAA and AMOH) were quite low, and AcAA appeared later than the other metabolites After intravenous administration of AM, the plasma concentration of MAA decreased as shown in Fig 6. As for oral administration, it was noticeable that AMCOOH, AcMAA, FAA, AMOH and $AMCH_2OH$ were detected in the plasma, though these levels were quite low

In summary, an improved assay for the determination of AM and its metabolites in the plasma of rats by HPLC has been developed This method is rapid, sensitive and amenable to the analysis of a small volume of plasma samples. Each assay requires about 120 min from the selection of plasma sample to the completion of assay. AM and its metabolites can be determined at levels as low as 0 02 μg/ml using 0 1 ml of plasma Recoveries of more than 97% indicate

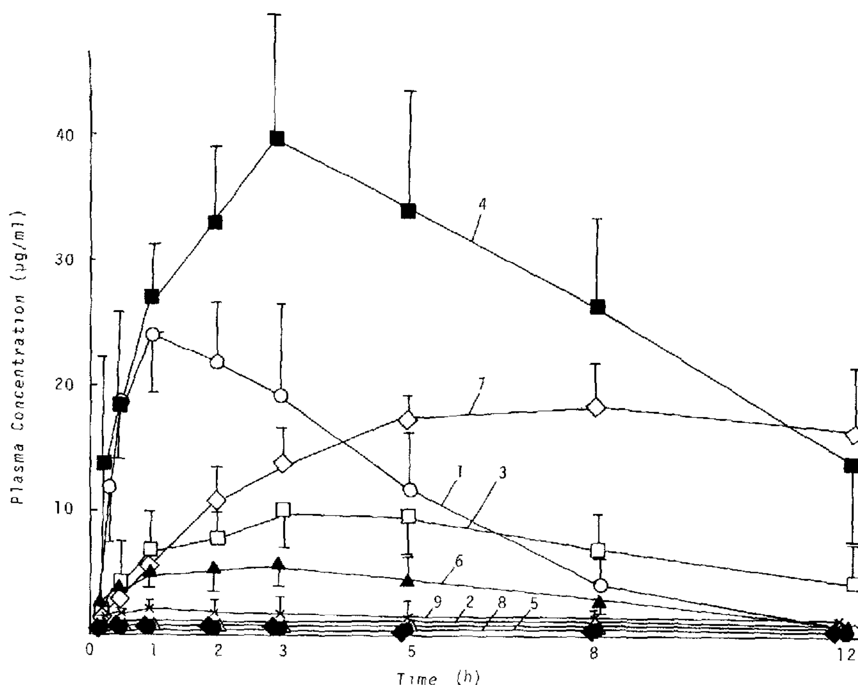


Fig 5 Plasma concentration—time curves of aminopyrine and its metabolites after oral administration of 100 mg/kg aminopyrine Each point represents the mean ± S D of ten experiments 1 = AM, 2 = AMOH, 3 = AA, 4 = MAA, 5 = AcMAA, 6 = $AMCH_2OH$, 7 = AcAA, 8 = FAA, 9 = AMCOOH

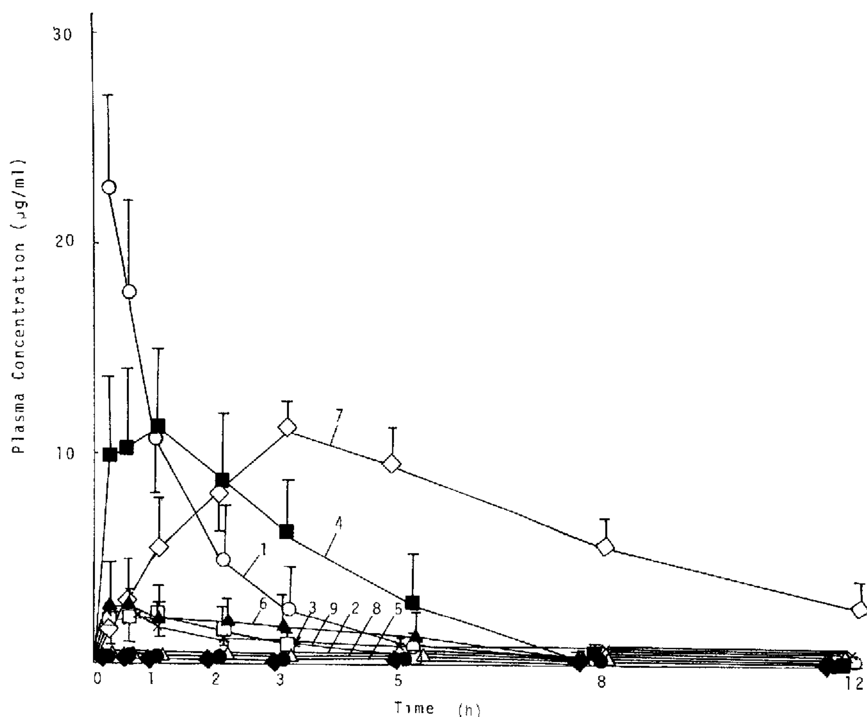


Fig. 6. Plasma concentration—time curves of aminopyrine and its metabolites after intravenous administration of 35 mg/kg aminopyrine Each point represents the mean ± S D of ten experiments 1 = AM, 2 = AMOH, 3 = AA, 4 = MAA, 5 = AcMAA, 6 = $AMCH_2OH$, 7 = AcAA, 8 = FAA, 9 = AMCOOH

that no component interfering with quantitative extraction is involved in the plasma Intra- and inter-assay repeatability studies have shown that the method is precise and reliable Our assay is an improvement over other methods for measuring these compounds in plasma samples We are currently using this method for pharmacokinetic studies of AM

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