Pergamon

0731-7085(93)E0008-B

Journal of Pharmaceutical & Biomedical Analysis, Vol. 12, No. 5, pp. 705–712, 1994

0731-7085/94 $7.00 + 0.00

A rapid and specific assay, based on liquid chromatography–atmospheric pressure chemical ionization mass spectrometry, for the determination of MK-434 (a 5α-reductase inhibitor) and its metabolites in plasma

T.V. OLAH,* J.D. GILBERT, A. BARRISH, T.F. GREBER and D.A. McLOUGHLIN

Merck Research Laboratories, West Point, PA 19486, USA

Abstract: MK-434 is a new 5α-reductase inhibitor. A sensitive and specific assay based on combined liquid chromatography–mass spectrometry (LC–MS) has been developed for the determination of this compound in plasma. The analyte was isolated from plasma by solid-phase extraction on a C_{18} cartridge. A related substance, L-654,066, was used as the internal standard. Extracts were separated on a 5-cm C_{18}-reversed-phase high performance liquid chromatography column interfaced via the heated nebulizer probe to a corona discharge chemical ionization source. The mass spectrometer was operated in the positive ion MS–MS mode. The method had sufficient sensitivity, precision, accuracy, and selectivity for the analysis of clinical samples containing MK-434 and its two principal metabolites at concentrations in the range 0.5–50 ng ml^{-1}. The chromatographic run time was <5 min.

Keywords: *MK-434; 5α-reductase inhibitor; plasma; analysis; LC–MS–MS; metabolites.*

Introduction

The enzyme 5α-reductase [1], which occurs in the prostate gland, converts testosterone to dihydrotestosterone [2–5]. The enzyme is inhibited by a series of 4-azasteroids [6–11] which are of potential value in the treatment of benign prostatic hyperplasia [12]. MK-434 (17β-benzoyl-4-aza-5α-androst-1-ene-3-one) is one of the latest azasteroids to be studied [13].

A sensitive and specific assay was required for the determination of MK-434 in biological fluids. The low volatility of MK-434 precluded its analysis by combined gas chromatography–mass spectrometry (GC–MS), and its *N*-trimethylsilyl and *N*-trifluoroacyl derivatives were unstable. Radioimmunoassay proved both convenient and sensitive but ultimately lacked specificity. High performance liquid chromatography (HPLC) with atmospheric pressure chemical ionization has previously proved highly successful for the convenient quantitation of drugs in body fluids [14–18]. This technique readily fulfilled the requisite assay criteria and permitted the simultaneous measurement of the diastereomeric alcohols resulting from metabolic reduction of the parent drug. A structural analogue, L-654,066 (5α-23-methyl-4-aza-21-nor-chol-1-ene-3,20-dione), was used as the internal standard.

Experimental

Materials

MK-434, L-691,919, L-694,579 and L-654,066 (Fig. 1) were synthesized at the Merck Research Laboratories (Rathway, NJ). Ammonium acetate, sodium acetate, disodium ethylenediaminetetra-acetic acid (EDTA), bovine serum albumin (BSA) and trifluoroacetic acid (TFA) were obtained from Sigma (St Louis, MO). Methanol (HPLC grade) and sodium phosphate (mono- and di-basic) were obtained from Fisher (Fair Lawn, NJ). Sodium hydroxide and acetic acid were of analytical grade and were purchased from Mallinckrodt (Paris, KT). SepPak-C_{18} cartridges (5 ml × 500 mg) were obtained from Waters (Milford, MA). Air (hydrocarbon-free), nitrogen (99.999%) and argon (99.999%) were purchased from Matheson (Morris Plains, NJ). Disposable tapered reaction vials (1 ml) and crimp caps were obtained from Rainin Instruments (Woburn, MA).

* Author to whom correspondence should be addressed.

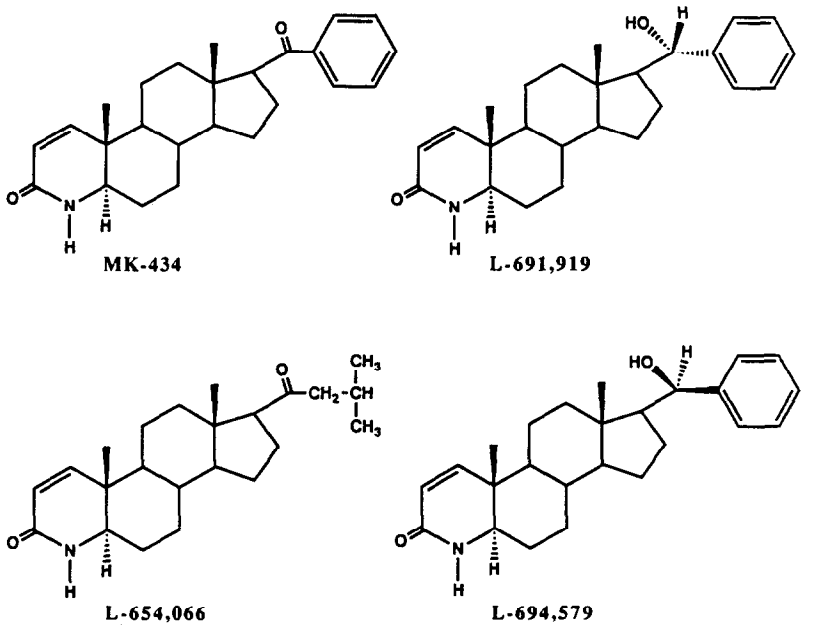

Figure 1
Structures of MK-434, its metabolites L-694,579 and L-691,919, and the internal standard L-654,066.

Standard and sample preparation

Standard stock solutions of MK-434, its metabolites (L-691,919 and L-694,579) and the internal standard (L-654,066) were prepared as 1 mg ml^{-1} solutions in acetic acid; 1.0 ml aliquots were diluted to 100.0 ml in methanol. Subsequent dilutions were prepared in a solution consisting of phosphate buffer (pH 7.5; 0.05 M) containing EDTA (0.05 M), 0.1% sodium azide, 0.05% BSA and ethanol (USP 200, 80:20, v/v) to prevent absorption of the analytes at low concentrations.

Standards and quality control samples were prepared by the addition of known amounts of standard solutions (0.1 ml) to 1.0 ml aliquots of control human plasma. The concentrations of MK-434 and its metabolites in standard plasma samples were generally 0.5, 1, 2, 5, 10, 20 and 50 ng ml^{-1}. Quality control samples were prepared at concentrations of 1, 5 and 20 ng ml^{-1}.

Extraction procedure

A plasma sample (1.0 ml) was placed in a 75 × 12 mm glass tube. After the addition of 0.1 ml of a 0.05 μg ml^{-1} solution of internal standard, the tube was briefly vortexed. The sample was applied to a SepPak cartridge, preconditioned by washing with methanol (4 ml) and distilled water (5 ml). The sample was drawn into the column (with vacuum applied) at approximately 1 ml min^{-1}. The column was washed with 3 ml of distilled water. After discarding the washings, the drug was eluted in methanol (3 ml) into a glass tube and the eluate reduced to dryness at 22°C in a Speed-Vac Model SVC200H (Savant, Farmingdale, NY). After extraction the following sample-transfer procedure was used to minimize adsorptive losses. The residue was transferred in ethanol (0.4 ml) into a micro-derivatization vial and the solvent removed in the SpeedVac. The residue was reconstituted in 0.1 ml of acetonitrile. The vial was capped, agitated in an ultrasonic bath for 20 min and briefly vortexed to remove air bubbles prior to analysis.

The recoveries of MK-434 and L-654,066 from plasma were determined in the following manner. Aliquots (1.0 ml) of control human plasma from five separate individuals were spiked with analyte or internal standard to yield concentrations of 2 ng ml^{-1}. After solid-phase extraction, 2 ng of L-654,066 was added to extracts containing MK-434, and 2 ng of MK-434 was added to extracts containing L-654,066. Samples were assayed by LC–MS–MS and the peak area ratios compared with those obtained from an unextracted reference solution (400 pg of both MK-434 and L-654,066). The recoveries of the metabolites were calculated subsequently from a comparison of the peak area ratios of sets of extracted and unextracted standards. The

absolute recoveries were MK-434 (95.0 ± 3.7%), L-654,066 (95.9 ± 1.8%), L-694,579 (93.2 ± 4.3%) and L-691,919 (92.5 ± 3.3%).

Chromatographic system suitability

The precision of the chromatographic system was determined by making 10 successive injections (20 μl) of a solution containing 0.06 μg ml^{-1} of MK-434, L-694,579, L-691,919 and the internal standard. The relative standard deviations (RSDs) of the peak area ratio of MK-434, L-694,579 and L-691,919 with respect to the internal standard were 6.27, 5.72, and 7.52%, respectively.

LC–MS–MS analysis

LC–MS–MS was performed on a Sciex (Thornhill, Ontario) Model API III triple quadrupole mass spectrometer interfaced via a Sciex heated nebulizer probe to a liquid chromatograph consisting of a Perkin Elmer 250 solvent delivery system and a Perkin Elmer ISS-100 autoinjector equipped with a 200 μl loop. Chromatography was performed using a column packed with SynChropak, C-18 (50 × 46 mm, i.d. 5 μm) from Sychrom Inc. (Lafayette, IN) and 20 μl aliquots of the extract were taken for analysis. The mobile phase consisted of acetonitrile–methanol–ammonium acetate (10 mM; 61.5:3.5:35, v/v/v) containing 0.1% (w/v) TFA at a flow rate of 1 ml min^{-1}. The nebulizer probe temperature setting was 500°C. The nebulizing gas (air) pressure and auxiliary flow were set at 80 p.s.i. and 2.0 l min^{-1}, respectively. Gas-phase chemical ionization was effected by a Corona discharge needle (+5 μA) and positive ions were sampled into the quadrupole mass analyser via a 0.0045″ pinhole aperture. The mass spectrometer was programmed to transmit the protonated molecules $[M + H]^+$ at *m/z* 378 (MK-434), *m/z* 380 (metabolites) and *m/z* 358 (internal standard) via the first quadrupole filter (Q1) with collision-induced fragmentation in Q2 (collision gas argon, 50 eV, 400 × 10^{12} atoms cm^{-2}) and monitoring, via Q3, the product ions at *m/z* = 310, 312 and 290 for MK-434, the metabolites and the internal standard, respectively. The orifice potential and electron multiplier settings were +80 V and −4.2 kV, respectively. The dwell time was 400 ms. Peak area ratios of each analyte with respect to the internal standard were computed using proprietary MacSpec software from Sciex. The calibration curves were constructed using non-weighted linear least-squares regressions of the plasma concentrations and the measured area ratios. The concentrations of MK-434 and its metabolites in test samples were determined by interpolation from the appropriate standard curve.

Radioimmunoassay

A radioimmunoassay for MK-434 in plasma was developed using rabbit antiserum raised against an immunogen prepared by coupling the hemisuccinate of the 11α-hydroxy derivative, via its *N*-hydroxy-succinimide ester, to BSA. Antiserum was diluted 1:20,000 in phosphate buffer (pH 7.5; 0.05 M) containing EDTA (0.05 M), 10 mg ml^{-1} BSA and 0.1 mg ml^{-1} sodium azide.

The radioligand was prepared by coupling the hemisuccinate of the 11α-hydroxy derivative, via its active ester, to ^{125}I-labelled tyrosine with subsequent purification by reversed-phase LC. The purified radioligand was reconstituted at a concentration of 200,000 cpm ml^{-1} in phosphate buffer containing sheep anti-rabbit globulin (Arnel Products, New York, NY). Extracts of test and standard plasma samples were prepared by solid-phase extraction as described above. The extract residues were reconstituted in phosphate buffer containing 20% ethanol. The radioimmunoassay was conducted in triplicate.

To 0.1 ml of reconstituted extract was added 0.1 ml of radioligand–second antibody (20,000 cpm), 0.1 ml of diluted antiserum–rabbit gamma globulin reagent and 0.7 ml of phosphate buffer. The binding at equilibrium was similar whether the primary and secondary antibodies were added simultaneously or sequentially. The mixture was incubated for 18 h at room temperature and subsequently centrifuged (800 ***g*** for 45 min). The supernatant was discarded and the radioactivity in the consolidated pellet was determined using a gamma counter (Micromedic, Horsham, PA). Samples were counted for 3 min. Calibration curves were constructed using a third-order polynominal, by plotting the fraction of control binding (B-NSB/B_o-NSB) against plasma concentration where B_o was the observed binding (cpm) in the absence of the analyte, B was the binding in the presence of a specific quantity of analyte, and NSB was the non-specific binding. The calibration range was 0.1–20 ng ml^{-1}. The concentrations of MK-434 in test samples were

determined by interpolation of the standard curve.

When evaluated by analysis of control (drug-free) plasma, to which MK-434 was added, the accuracy (found/added) of the radioimmunoassay at 0.2, 2 and 20 ng ml^{-1} was 104, 102 and 102%, respectively, with corresponding intra-day RSDs (n = 5) of 4.9, 5.4 and 2.1%. The non-specific binding was 1.4%. When applied to plasma from patients to whom MK-434 had been administered, parallelism and recovery experiments were satisfactory.

Results and Discussion

The principal objective was the development of an assay capable of measuring MK-434 in plasma at concentrations in the range 0.5–50 ng ml^{-1}. The ease with which the metabolites could be determined concomitantly was an advantage, unanticipated at the commencement of the assay development program. The product ion mass spectra of MK-434, its metabolites, and the internal standard showed diagnostic fragments at m/z 310, 312 and 290, respectively (Fig. 2). The spectra of L-694,579 and L-691,919 are essentially identical. Chromatograms, using MS–MS detection, of extracts of plasma from a volunteer receiving an oral dose (25 mg daily) of MK-434 are shown in Fig. 3.

Calibration

Typical calibration curves for the analyte and its metabolites are shown in Fig. 4. The data demonstrate acceptable accuracy, reproducibility, and good fit to the non-weighted regression lines (Table 1). Calibration standards of plasma containing neither drug nor metabolites showed peak area ratios that were essentially zero. There was no interference from endogenous plasma components, with the determination of the parent drug, its metabolites, or the internal standard.

Precision and accuracy

Assay accuracy and intra-day precision were determined by analysis of replicates (n = 5) of control plasma containing known concentrations of MK-434, L-694,579 and L-691,919 over the concentration range from 0.5 to 50 ng ml^{-1}. The RSDs at the lowest concentration (0.5 ng ml^{-1}) were 8.1, 6.6 and 17.4%, for MK-434, L-694,579 and L-691,919, respectively. The criterion for the acceptance of

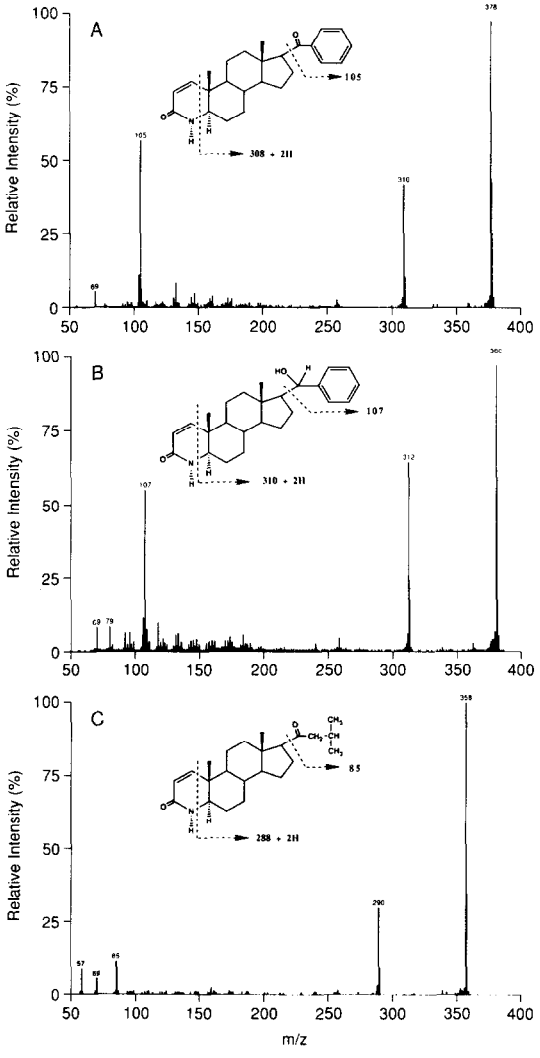

Figure 2
Positive product ion mass spectra (background subtracted) of the protonated molecular ions of (a) MK-434 (m/z 378), (b) L-691,919 (m/z 380) and (c) the internal standard L-654,066 (m/z 358). The mass spectrum of L-691,919 is identical to that of its diastereomer L-694-579.

clinical data was that intra-assay RSD should not exceed 10%. As a result, the limit for reliable determination of L-691,919 in plasma using the present calibration range was defined as 1 ng ml^{-1}. Inter-day assay precision was calculated from quality control data obtained during successive analyses. Results are shown in Table 2. Acceptable accuracy, defined as (mean % found/added), intra- and inter-day precision were demonstrated.

Method applications

The lack of specificity of the radioimmunoassay, when applied to human samples, was demonstrated by LC fractionation of extracts

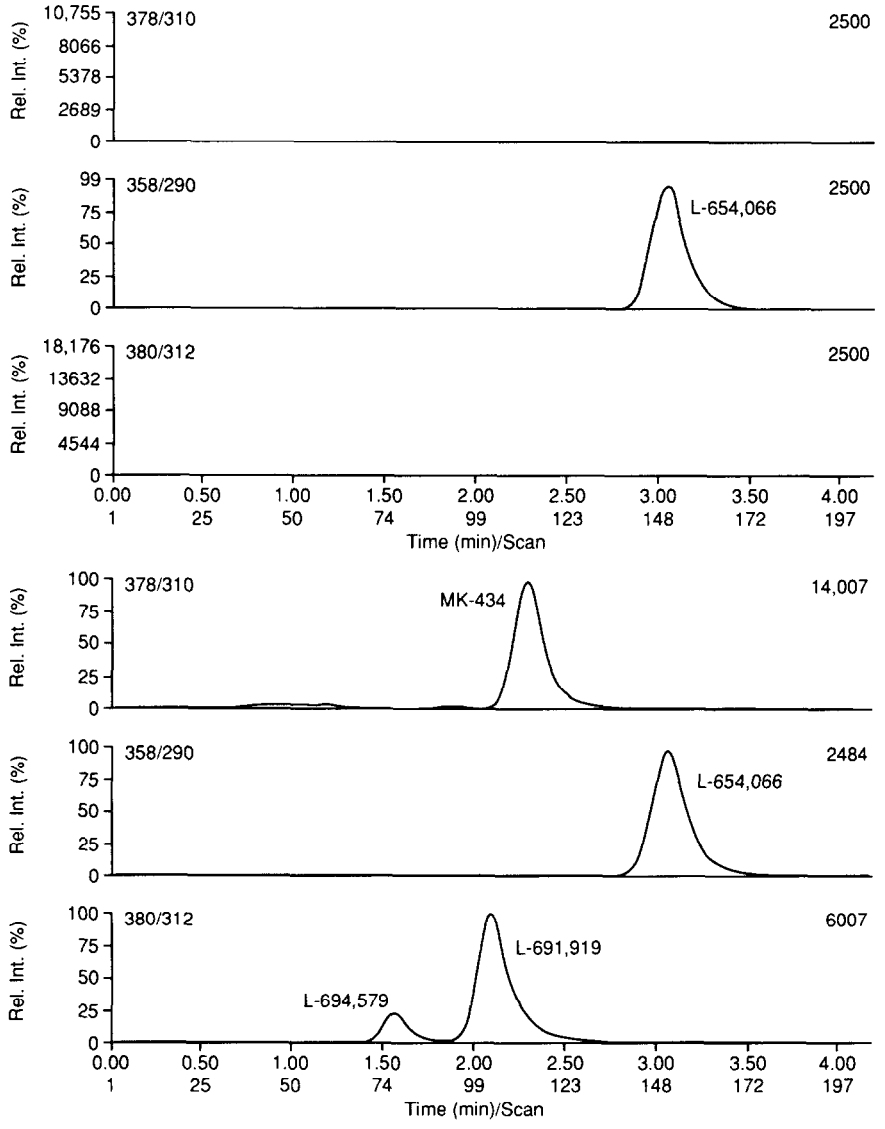

Figure 3
Chromatograms obtained by multiple reaction monitoring of extracts of plasma from a volunteer receiving 25 mg (p.o.) of MK-434. Retention times are shown in minutes. Channel A, *m/z* 378 → 310, MK-434; Channel B, *m/z* 358 → 290, internal standard: and Channel C, *m/z* 380 → 312, L-694,579 and L-691,919. Top: extract of a plasma sample collected prior to drug administration. Bottom: extract of a plasma sample, collected 6 h after administration containing 25 ng ml^{-1} MK-434, 15.8 ng ml^{-1} L-691,919 and 3.7 ng ml^{-1} L-694,579.

Table 1
Mean peak area ratios and curve parameters determined during the construction of five successive calibration lines for the determination of MK-434 and its metabolites L-694,579 and L-691,919 in plasma

	MK-434		L-694,579		L-691,919	
Conc. plasma (ng ml^{-1})	Mean	RSD (%)	Mean	RSD (%)	Mean	RSD (%)
0.5	0.110	6.5	0.051	5.6	0.053	15.2
1	0.203	4.9	0.101	2.1	0.102	8.0
2	0.386	4.7	0.203	3.6	0.188	6.7
5	0.985	4.0	0.505	3.4	0.474	6.7
10	1.910	2.6	1.050	7.3	0.925	4.7
20	3.890	3.8	2.140	8.5	1.890	10.1
50	9.180	4.7	4.930	4.5	4.410	5.8
Slope	0.184	4.3	0.099	4.1	0.088	5.7
Intercept	0.047	82.3	0.027	135.1	0.030	82.4
r^2	0.999	0.13	0.997	0.34	0.997	0.23

Table 2
Intra- and inter-day accuracy and precision for the determination of MK-434 and its metabolites L-694,579 and L-691,919 in plasma by LC–MS–MS

		MK-434		L-694,579		L-691,919	
	Actual conc. (ng ml^{-1})	Accuracy (mean % found/added)	Precision (RSD %)	Accuracy (mean % found/added)	Precision (RSD %)	Accuracy (mean % found/added)	Precision (RSD %)
Intra-day	0.5	94.0	8.1	92.0	6.6	92.0	17.4
(n = 5)	1	98.0	4.3	97.0	1.2	100.0	8.5
	2	96.5	5.1	100.0	3.7	98.5	6.9
	5	100.4	4.9	101.4	3.4	101.2	6.8
	10	100.0	2.6	106.0	7.4	99.3	4.7
	20	102.5	3.8	108.5	8.6	102.0	10.1
	50	97.0	4.7	99.8	4.4	95.4	5.8
Inter-day	1	109.0*	7.8*	98.1	7.2	95.0	12.4
(n = 5)	5	100.9*	6.1*	104.0	2.5	104.0	13.4
	20	101.3*	7.0*	102.1	4.5	105.0	7.8

*n = 15.

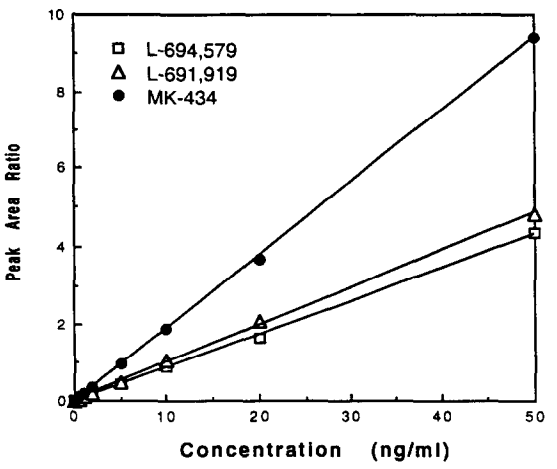

Figure 4
Calibration curves for the determination of MK-434 and its metabolites in plasma.

of plasma obtained from subjects participating in clinical studies. Overestimation of the drug concentration by two- to three-fold was observed. The detection of immunoreactive substances other than the parent drug suggested the presence of cross-reacting metabolite(s). The principal metabolite (L-691,919), which resulted from reduction of the ketone to an alcohol, did not show appreciable reactivity. A comparison of the concentrations of MK-434 in plasma determined by both radioimmunoassay and LC–MS–MS after a single oral dose (25 mg) to volunteers is shown in Table 3. The results confirmed the lack of specificity of the immunoassay, but the source of the interference was unknown.

Conclusions

The present assay was established specifi-

Table 3
Comparison of the concentrations of MK-434 (ng ml^{-1}) determined by both radioimmunoassay and LC–MS–MS in the plasma of two volunteers receiving a single oral dose of 25 mg

Time post-dose (h)	Subject 1			Subject 2		
	RIA	LC–MS–MS	Ratio RIA–MS	RIA	LC–MS–MS	Ratio RIA–MS
0	<0.1	<0.5		<0.1	<0.5	
2	5.7	2.4	2.4	6.5	2.9	2.2
4	12.9	6.5	2.0	6.3	3.2	2.0
8	10.2	4.6	2.2	5.4	1.7	3.2
12	7.7	3.4	2.3	5.9	2.3	2.6
24	9.8	3.7	2.6	5.9	2.7	2.2
48	5.5	1.8	3.1	3.1	1.3	2.4

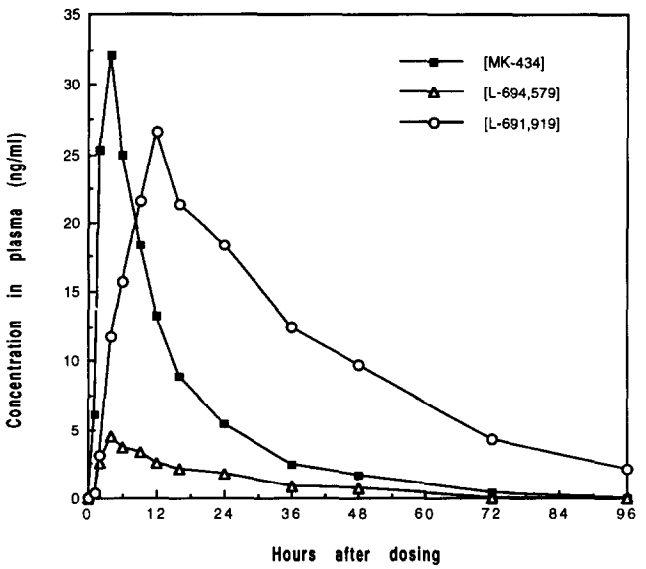

Figure 5
Plasma concentration–time profiles of MK-434 and its metabolites in the plasma of a subject receiving 25 mg of the drug.

cally to determine MK-434 in plasma at concentrations in the range 0.5–50 ng ml^{-1}. During the course of development of the assay, its ready extension to the simultaneous quantitation of the diastereomeric products of metabolic reduction became apparent. Since the recoveries from solid-phase extraction were comparable with those of MK-434 and its internal standard, the assay was readily adapted to encompass the assay of all three substances.

The versatility of the Sciex heated-nebulizer interface in conjunction with triple quadrupole LC–MS–MS is gaining wide acceptance in current bioanalytical research. Particular attractions are the capacity for flow rates in excess of 1 ml min^{-1} and the high specificity of the technique which enable the use of fast liquid chromatographic separations in conjunction with automated sample injection and data acquisition. In the present assay, the retention time of MK-434 is approximately 2.2 min and the analysis of the drug and its metabolites can be completed in 5 min. The linearity of the detector response was satisfactory over two orders of magnitude and enabled the use of a simple non-weighted linear regression.

This method has been applied to the determination of MK-434 and two of its metabolites in plasma obtained from clinical studies. The plasma concentration–time curves, obtained by analysis of samples collected from a subject receiving a single oral dose of MK-434 are shown in Fig. 5.

Acknowledgements — The authors thank Dr G.H. Rasmusson for the synthesis of the derivative used in the development of the radioimmunoassay and Ms P.C. Schaible for her assistance in the preparation of this manuscript.

References

[1] P. Ofner, I. Leav and L.F. Cavazos, in *Male Accessory Sex Organs* (D. Brandes, Ed.), pp. 267–289. Academic Press, New York (1974).
[2] N. Bruchovsky and J.D. Wilson, *J. Biol. Chem.* **243**, 2012–2021 (1968).
[3] K.M. Anderson and S. Liao, *Nature* **219**, 277–279 (1968).
[4] G.C. Haltmeyer and K.B. Eik-Nes, *Acta Endocrinol.* **69**, 394–402 (1972).
[5] E.P. Giorgi, J.C. Stewart, J.K. Grant and R. Scott, *Biochem. J.* **123**, 41–55 (1971).
[6] G.H. Rasmusson, G.F. Reynolds, T. Utne, R.B. Jobson, R.L. Primka, C. Berman and J.R. Brooks, *J. Med. Chem.* **27**, 1690–1701 (1984).
[7] T. Liang, G.H. Rasmusson and J.R. Brooks, *J. Steroid Biochem.* **19**, 385–390 (1983).
[8] T. Liang, M.A. Cascieri, A.H. Cheung, G.F. Reynolds and G.H. Rasmusson, *Endocrinology* **117**, 571–579 (1985).
[9] T. Liang, C.E. Heiss, A.H. Cheung, G.F. Reynolds and G.H. Rasmusson, *J. Biol. Chem.* **25**, 734–739 (1984).
[10] J.R. Brooks, C. Berman, R.L. Primka, G.F. Reynolds and G.H. Rasmusson, *Steroids* **47**, 1–19 (1986).
[11] P.J. De Schepper, J. Imperato-McGinley, A. Van Hecken, I. DeLapeleire, A. Buntinx, J. Carlin, M.H. Gressi and E. Stoner, *Steroids* **56**, 469–471 (1991).
[12] E. Stoner, *J. Steroid Biochem. Molec. Biol.* **37**, 375–378 (1990).
[13] S.M. Cohen, J.G. Werrmann, G.H. Rasmusson, W.K. Tanaka, P.F. Maleteska, S. Prahalada and J.G. Jacobs, *Prostate.* Submitted.
[14] T.R. Covey, E.D. Lee and J.D. Henion, *Anal. Chem.* **58**, 2453–2460 (1986).
[15] H. Fouda, M. Nocerini, R. Schneider and C. Gedutis, *J. Am. Soc. Mass Spectrom.* **2**, 164–167 (1991).
[16] J.D. Gilbert, E.L. Hand, A.S. Yuan, T.V. Olah and T.R. Covey, *Biological Mass Spectrom.* **21**, 63–68 (1992).
[17] M.J. Avery, D.Y. Mitchell, F.C. Falkner and H.G. Fouda, *Biological Mass Spectrom.* **21**, 353–357 (1992).
[18] J.D. Gilbert, T.V. Olah, A. Barrish and T.F. Greber, *Biological Mass Spectrom.* **21**, 341–346 (1992).

[Received for review 6 July 1993; revised manuscript received 24 September 1993]