ELSEVIER

Journal of Chromatography B, 772 (2002) 89–98

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Rapid pharmacokinetic screening for the selection of new drug discovery candidates using a generic isocratic liquid chromatography–atmospheric pressure ionization tandem mass spectrometry method

Lawrence F. Colwell Jr.*, Constantin S. Tamvakopoulos, Pei Ran Wang, James V. Pivnichny, Thomas L. Shih

Department of Basic Chemistry, Merck Research Laboratories, RY800B-206, P.O. Box 2000, Rahway, NJ 07065, USA

Received 18 July 2000; received in revised form 18 January 2002; accepted 25 January 2002

Abstract

A generic isocratic HPLC–APCI-MS–MS method has been developed for the determination of plasma concentrations of bioactive compounds for the selection of potential new drug discovery candidates. A 4.6×50 mm cyano phase column eluted with an acetonitrile/water mobile phase containing 20 m*M* ammonium acetate and 0.4% TFA produces retention times of 1 min or less for a wide range of compounds. This is a great advantage in new drug discovery where many compounds are analyzed once and eliminated. No time is consumed developing chromatographic conditions for each new compound. The mass spectrometer can be optimized and the samples can be processed and analyzed, all in the same day. Multiple assays can be run consecutively without changing the column or mobile phase between assays.

Keywords: Drug discovery candidates

1. Introduction

Recent trends in medicinal chemistry have led toward the acquisition of pharmacokinetic data in the earliest stages of drug development. Chemists use bioactivity, as well as other PK data to direct their synthesis. Typically, compounds are evaluated for potency and specificity using in vitro biological assays. Selected compounds are further evaluated in vivo in an animal model to determine if they remain in circulation at a concentration that will produce the desired effect. Rapid turn-around is required so that medicinal chemists can direct their efforts toward the synthesis of compounds that have the potential for further development. A common problem encountered in the early stage of drug discovery is low bioavailability in the range of 5% or less. In these cases, the chemists are usually looking for gross changes in oral absorption. A simple three time point oral dose assay in rodents will often provide the information needed to determine if it is worthwhile to scale-up a synthesis to provide enough material for a full PK study.

In supporting these types of studies, many differ-

*Corresponding author. Fax: +1-732-594-9545.

1570-0232/02/$ – see front matter
PII: S1570-0232(02)00061-2

ent types of compounds are assayed on a daily basis. A method of analysis must be of general application and still be specific for a given compound. LC–MS–MS allows for the quick development of bioanalytical methods that are fast, sensitive and reliable.

Since its introduction in 1986 by Covey et al. [1], high-performance liquid chromatography atmospheric pressure ionization tandem mass Spectrometry (HPLC–API-MS–MS) has been proven to be a highly useful tool for the quantitation of pharmacological compounds in physiological fluids [2–7]. The high sensitivity and selectivity of this technique allows for the rapid development of quantitative assays without the need for extensive sample preparation. Of the two ionization techniques commonly employed in API-MS–MS, atmospheric pressure chemical ionization (APCI) is most easily adapted for the HPLC analysis of pharmaceutical compounds in crude extracts. There are several characteristics of the electrospray ionization (ESI) interface that make it less than ideal for the stated application.

ESI works best for compounds that are ionic in solution. Factors such as the pK_a of the analyte, the presence of other electrolytes in solution and the pH of the mobile phase can all have a great affect on the ion signal observed under ESI conditions. Loss of analyte ion intensity has been associated with the presence of ionizable components in the sample matrix and the mobile phase [8–12]. Variable amounts of interfering compounds in biological fluids can adversely affect the precision of a quantitative ESI-MS–MS assay [13]. In contrast, APCI is a gas phase process that is not limited by chromatographic conditions. It is not necessary to separate the analyte from components that elute at or near the solvent front.

The heated nebulizer interface used for APCI produces gas phase chemical ionization of compounds eluting from HPLC columns under normal elution conditions. It is more tolerant of mobile phase modifiers that could foul an ESI interface or suppress the ESI signal.

Reported here is a generic HPLC–APCI-MS–MS method that can be applied to the analysis of a wide range of compounds with no modification of the mobile phase. Analytes are detected in 100-μl plasma samples in a range between 1 ng/ml and 1 μg/ml with chromatographic run times of 2 min or less.

2. Experimental

2.1. Chemicals

All analytes were obtained from Merck Research Laboratories (Rahway, NJ, USA). HPLC grade acetonitrile (Fisher Scientific, Fair Lawn, NJ, USA), HPLC grade ammonium acetate (Fisher Scientific, Fair Lawn, NJ, USA), 99+% trifluoroacetic acid (Aldrich, Milwaukee, WI, USA) and dog serum (Pel-Freez Biologicals, Rogers, AK, USA) were all purchased from their respective vendors. Distilled water passed through a Milli-Q water system (Millipore, Belford, MA, USA) was used for all aqueous solutions. Mobile Phase was passed through a 0.45-μm nylon 66 membrane (Altech Associates, Deerfield, IL, USA) prior to use. Vacuum centrifugation was carried out with a Savant SpeedVac AES 2000 vacuum centrifuge (Savant Instruments, Farmingdale, NY, USA). Sample agitation was carried out using either a Fisher FS-14, a Fisher Model 361 orbital shaker or a Fisher Vortex Genie II (Fisher Scientific, Fair Lawn, NJ, USA).

2.2. Standard Solutions

Stock solutions of standards were prepared at 1.0 mg/ml in 50% acetonitrile/water. From this, standard solutions were made in 50% acetonitrile/water at the following concentrations: 8 μg/ml, 4 μg/ml, 2 μg/ml, 800 ng/ml, 400 ng/ml, 200 ng/ml, 80 ng/ml, 40 ng/ml, 20 ng/ml, 8 ng/ml and 4 ng/ml. Stock solutions of internal standard were prepared at 1.0 μg/ml in 50% acetonitrile/water. Standards were prepared by adding 25 μl of stock standards to 100 μl of plasma or serum to yield nominal concentrations of 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, 2000 ng/ml, relative to the plasma volume. A 25-μl aliquot of internal standard was added to all standards.

2.3. Sample preparation

A 25-μl aliquot of 50% acetonitrile/water and a 25-μl aliquot of internal standard was added to 100 μl of sample and vortex-mixed. Prior to analysis, samples and standards were either precipitated with acetonitrile or extracted with ethyl acetate. The

resulting supernatant was brought to dryness and reconstituted in mobile phase.

2.4. Protein precipitation

Acetonitrile (0.6 ml) was added to the samples and standards in 13×100 mm borosilicate tubes and vortex-mixed. After 5 min in a centrifuge at 1100 rpm, the precipitated proteins were fixed to the glass wall of the tube where they remained when the supernatants were transferred to 13×100 mm borosilicate tubes and evaporated to dryness using a Savant SpeedVac AES 2000 vacuum centrifuge. Dried samples were reconstituted in 200 μl of mobile phase, vortex-mixed, sonicated in a Fisher FS-14 ultrasonic agitator for 5 min and transferred to auto sampler vials with low volume micro inserts.

2.5. Extraction

A 0.2-ml aliquot of 0.1 *M* sodium carbonate (pH 10) was added to all tubes and vortex-mixed. After the addition of 4 ml of ethyl acetate, caps were placed on the tubes which were then secured in a test-tube rack using a second rack and a bungee cord. The rack of samples was placed sideways on a Fisher Model 361 orbital shaker and agitated at 300 cps for 5 min. The tubes were placed in a centrifuge for 5 min at 1100 rpm, the ethyl acetate layer was transferred to 13×100 mm borosilicate tubes and evaporated in the Savant SpeedVac AES 2000 and processed as above.

2.6. HPLC–MS–MS

A Waters Model 600 MS HPLC system and a Model 717 autosampler (Waters, Milford, MA, USA) were used for all HPLC analyses. Chromatography was carried out using a Phase Separations Spherisorb 4.6×50 mm S5CN cartridge column (Bodman Industries, Aston, PA, USA) with a 0.5-μm prefilter (Upchurch Scientific, Oak Harbor, WA, USA). The mobile phase consisted of a 60:40 mixture of acetonitrile–water containing 20 m*M* ammonium acetate and 0.4% trifluoroacetic acid delivered at a flow-rate of 1.0 ml per min. The effluent from the HPLC column was directed into the Atmospheric Pressure Chemical Ionization (APCI) interface of a PE Sciex API III+ triple quadrupole mass spectrometer (PE SCIEX, Concord, Ont., Canada) operated in the positive ion mode with the probe temperature set at 550 °C. The pressure of the nebulizer air was set at 80 p.s.i. with the nebulizer and auxiliary flows set to 1.2 and 2.0 ml per min, respectively. Nitrogen curtain gas was set at 1.2 ml per min. The source housing was kept at a vacuum of 1.2 inches of water to prevent condensation on the interface. The orifice temperature was maintained at 60 °C with the potential set at +55 V. MS–MS and multiple reaction monitoring (MRM) analyses were carried out using argon as the collision gas at a thickness of 2.34×10^{12} molecules per cm^2. The mass spectrometer was set to pass protonated molecular ions $[M+H]^+$ through the first quadrupole (Q1). Fragment ions produced via collision in (Q2) were isolated and detected using Q3. The resulting signal was processed with a Macintosh computer using Sciex software. Data collection and analysis were carried out using the RAD and MacQuan programs.

2.7. Procedure

A full scan positive ion spectrum of each compound was obtained to determine the molecular ion. Several MRM parent/product ion pairs were selected from each MS–MS spectrum for optimization of the collision energy. The autosampler was set for sequential injections with a zero run time. The cycle time (~35 s) of the autosampler produced a gap between peaks in which the collision energy was adjusted. Optimum conditions were determined by changing the collision energy in increments of 5 V in the range +5 V above to −10 V below the default setting (R2=0). The MRM pair that produced the most intense signal was selected and further optimized by varying the collision energy ±2 V around the voltage that produced the greatest response. Assay injection volume was determined by measuring the area counts for a 10-μl injection of the 4 ng/ml standard. An internal standard was selected that was structurally similar and possessed a unique MRM transition that neither interfered with nor was interfered by the MRM transition of the analyte. Sequential injections of the analyte, the internal standard and processed blank plasma were made to check for interferences.

3. Results and discussion

Seven compounds (Fig. 1) representing a range of molecular mass and polarity were used to optimize HPLC and APCI-MS–MS conditions.

3.1. Retention time optimization

The effect of the various mobile phase modifiers on solute retention are summarized in Table 1. The widest range of retention time values was obtained at neutral pH with 10 m*M* ammonium acetate. Enapril, finasteride and losartan eluted unretained while indinavir eluted at a retention time of 4.8 min. Neither ivermectin nor lovastatin produced a protonated molecular ion, both were detected as ammonium adduct ions, $[M+NH_4]^+$. The addition of 0.1% acetic acid produced some improvement, but the range of retention times was still larger than desired and lovastatin eluted as a split peak. Having observed the effect of the acetic acid, a lower pH was tried using 0.1% TFA as the sole modifier. It had the desired effect, eluting all seven compounds in a retention time range between 0.78 and 0.85 min. Under these conditions, the protonated molecular ion of both ivermectin and lovastatin were in the form of the sodium adduct, producing an unacceptably low signal for lovastatin (Table 2). A modifier of 10 m*M* ammonium acetate with 0.1% TFA restored the signal intensity for ivermectin to almost 70% of that

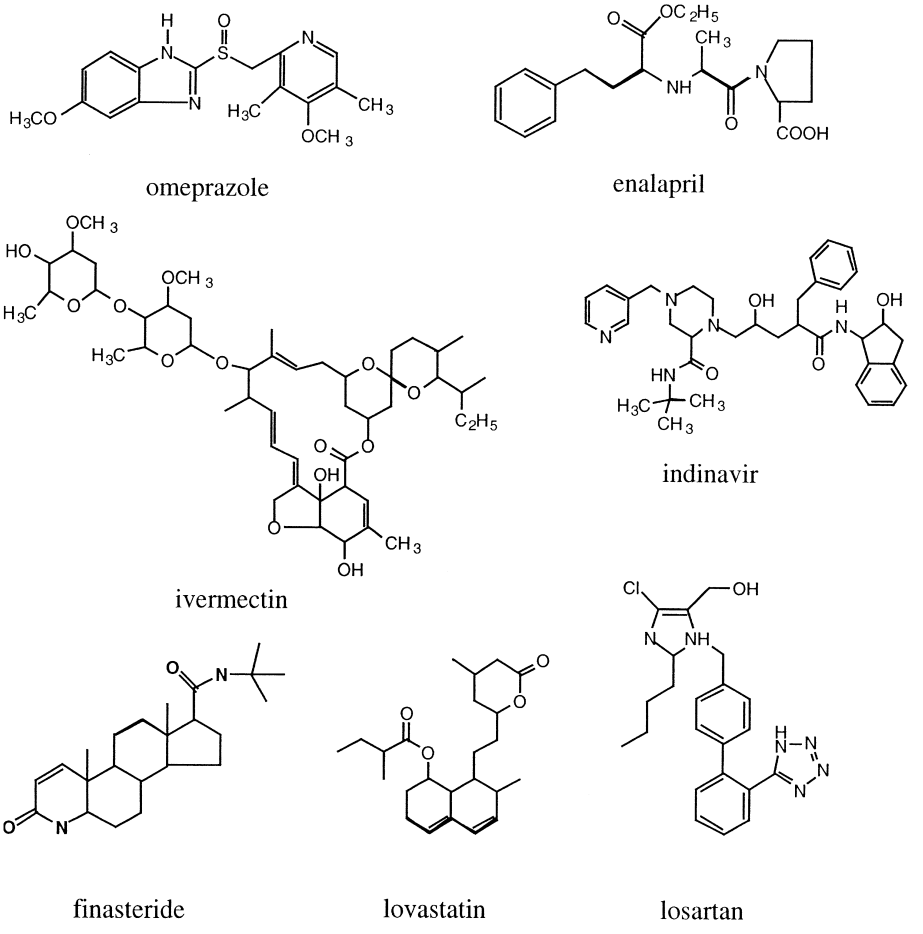

Fig. 1. Structure of enalapril, finasteride, indinavir, ivermectin, losartan, lovastatin and omeprazole.

Table 1
Effect of mobile phase modifier on retention time (min) using a 4.6×50 mm SCN column with a mobile phase of 60% acetonitrile/water at 1.0 ml/min

Compound	Mobile phase modifier				
	10 m*M* NH_4OAc	10 m*M* NH_4OAc 0.1% HOAc	0.1% TFA	10 m*M* NH_4OAc 0.1% TFA	20 m*M* NH_4OAc 0.4% TFA
Indinavir	4.80	1.35	0.85	1.37	0.81
Omeprazole	0.76	0.76	0.78	0.94	0.76
Finasteride	0.82	0.82	0.84	0.83	0.86
Enalapril	0.47	0.66	0.80	0.99	0.79
Lovastatin	0.48	0.63	0.80	0.72	0.79
Losartan	0.45	0.59	0.81	0.90	0.81
Ivermectin	0.82	0.79	0.85	0.81	0.83

obtained with the acetic acid/ammonium acetate combination. However, the retention time for indinvar was once again outside the desired range. After further optimization, a mobile phase of 20 m*M* NH_4OAc with 0.4% TFA in 60% acetonitrile/water was found to produce a usable signal with a retention time of less than 1 min for all seven compounds. Extracted ion chromatograms of the test compound eluted with final mobile phase are presented in Fig. 2. The low pH produced by the TFA minimizes the interaction of basic compounds with exposed silinols on the stationary phase and suppresses the ionization of acids. The net effect on a cyano column is that most compounds of interest elute within a narrow range of retention times.

Although acceptable chromatographic response is exhibited by all of the test compounds, it does not mean that they are suited for this method of analysis. Other factors must be considered. For example, lovastatin is converted by the liver to the active β-hydroxy acid metabolite. Direct detection of both of these compounds by APCI is not feasible. Wu et al. [14] have developed an ionspray method that incorporates positive and negative ion switching to analyze both components in a single chromatographic run. A second consideration is the possibility of the co-elution of an unstable metabolite, such as an *n*-oxide, which could interfere with the analyte signal if it were converted back to the parent compound by the heated nebulizer. For the purpose of screening compounds that are poorly absorbed or have a high first-pass effect, interference is not a major issue since these types of interference would result in exaggerated bioavailability. The vast majority of compounds tested do not produce plasma concentrations high enough to warrant further consideration.

Table 2
Effect of mobile phase modifier on ratio of MRM signal strength relative that obtained with 10 m*M* ammonium acetate using a 4.6×50 mm SCN column with 60% acetonitrile/water at 1.0 ml/min

Compound	Relative response				
	10 m*M* NH_4OAc	10 m*M* NH_4OAc 0.1% HOAc	0.1% TFA	10 m*M* NH_4OAc 0.1% TFA	20 m*M* NH_4OAc 0.4% TFA
Indinavir	1.0	3.52	0.08	3.51	1.64
Omeprazole	1.0	1.24	0.3	1.01	0.68
Finasteride	1.0	1.54	1.0	1.18	0.55
Enalapril	1.0	1.42	0.25	0.71	0.44
Lovastatin	1.0	0.70	0.14	0.54	0.47
Losartan	1.0	0.81	0.20	0.50	0.62
Ivermectin	1.0	2.26	0.003	1.48	1.08

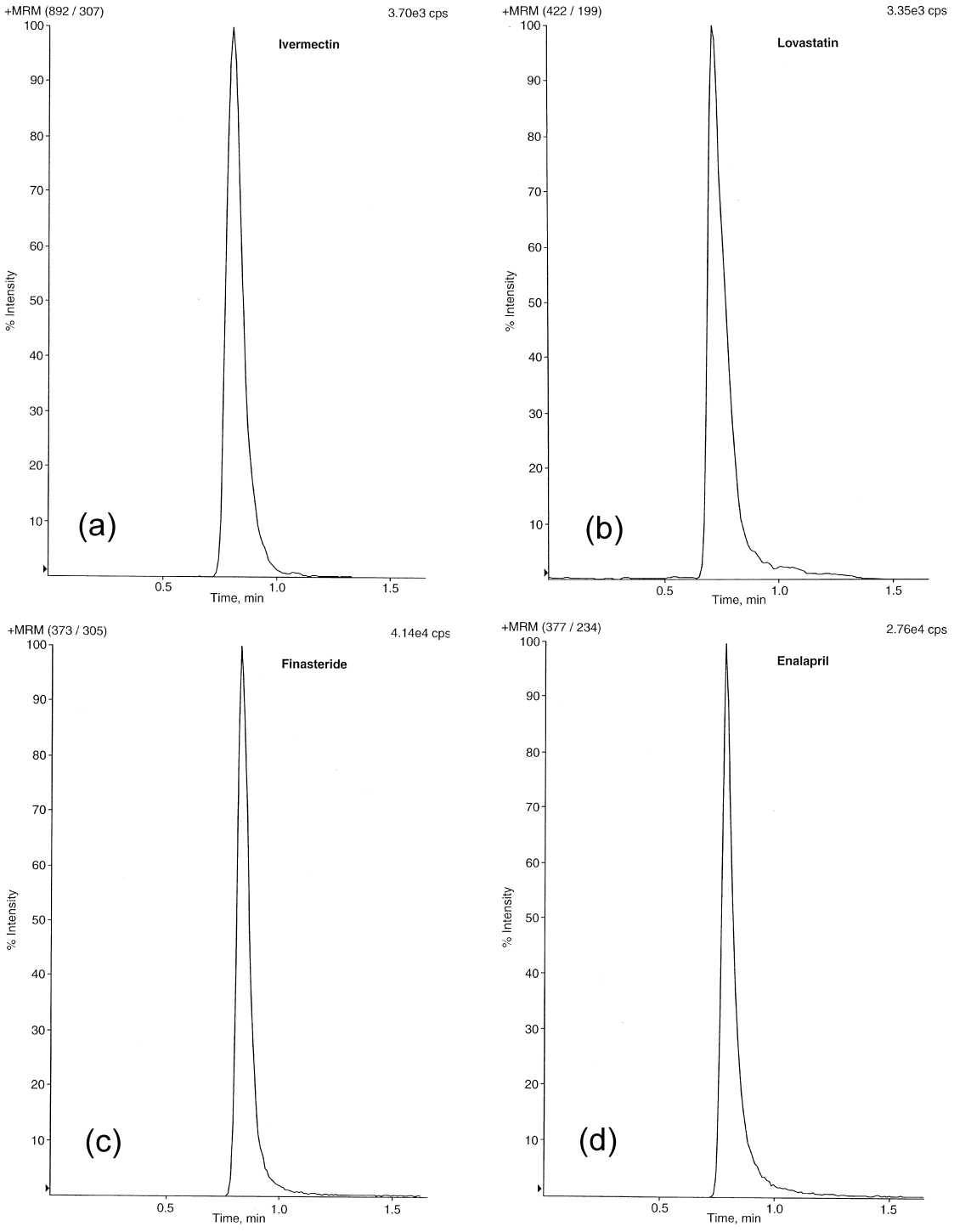

Fig. 2. Extracted ion chromatograms of the seven test compounds analyzed on a 4.6×50 mm cyano phase column with a mobile phase of 20 m*M* NH_4OAc with 0.4% TFA in 60% acetonitrile/water.

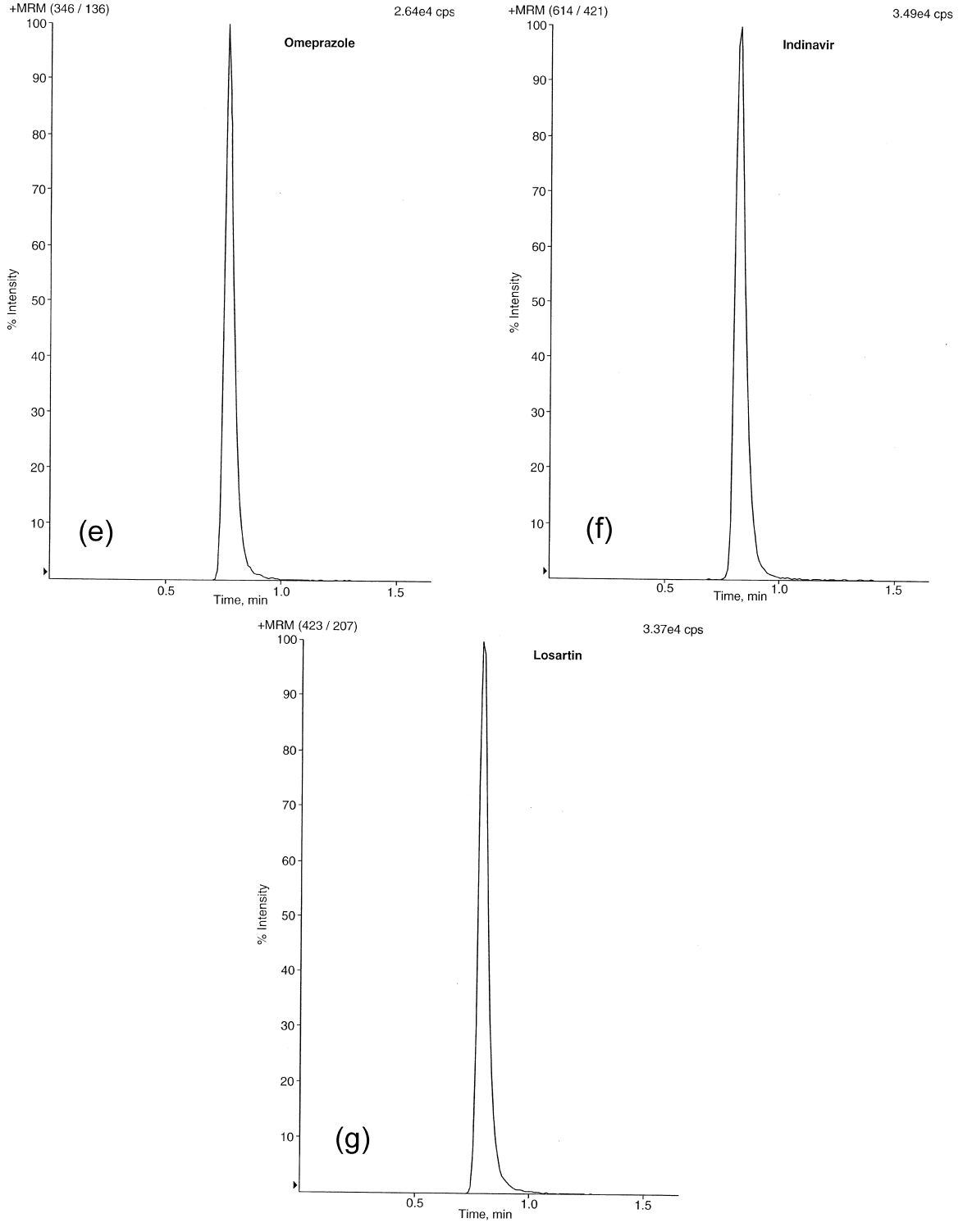

Fig. 2. (*continued*)

Compounds that produce appropriate plasma levels are subjected to further testing using methods that offer a higher degree of separation.

3.2. Analysis of L-770,644

An example of the utility of this method was demonstrated in the analysis of L-770,644, a selective β_3 adrenergic receptor agonist [15]. L-770,644 was analyzed in extracted dog plasma samples using a structurally related compound as an internal standard. The structure and MS–MS product ion spectrum for each compound are shown in Fig. 3. The protonated molecular ions [MH^+] of L-770,644 and the internal standard are at m/z 592 and 569, respectively. Product ions at m/z 439 and 386 from

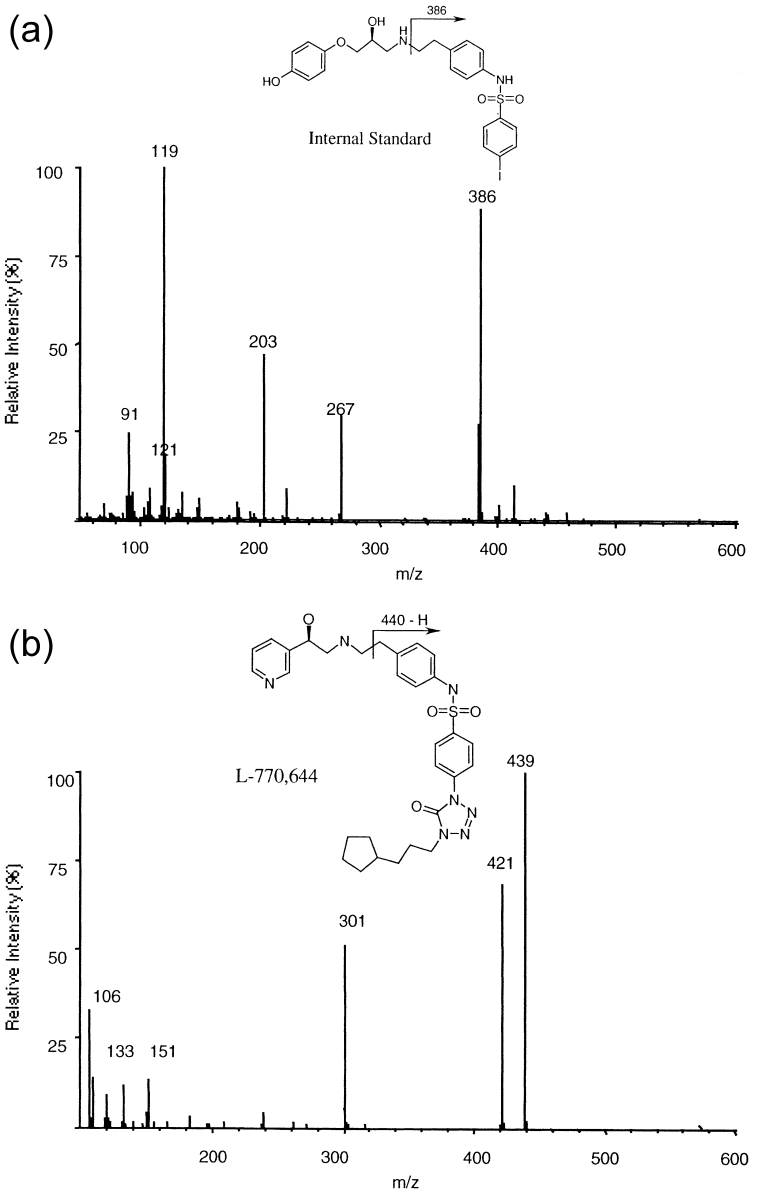

Fig. 3. Product ion spectrum of the internal standard and L-770,644.

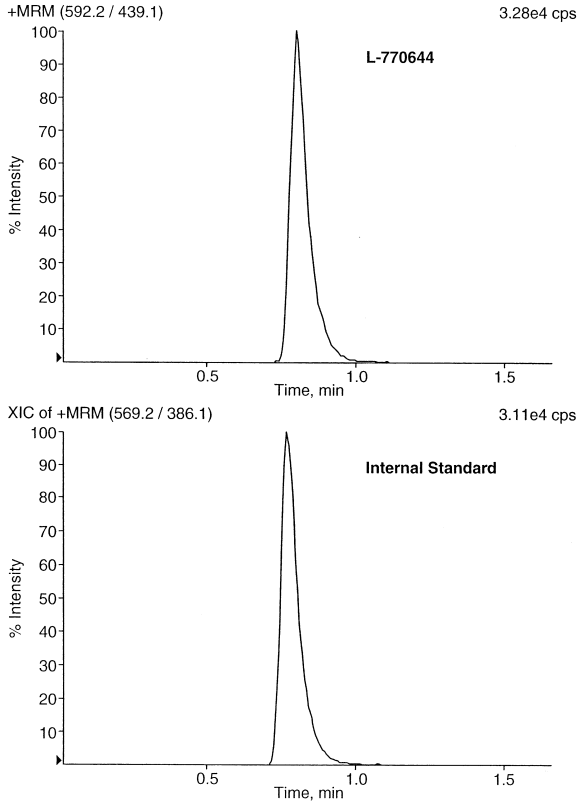

Fig. 4. Extracted ion chromatograms of L-770,644 and the internal standard.

Table 3
Intra-day precision[a] and accuracy of the assay of L-770,644 in dog plasma

Nominal concentration (ng ml^{-1})	Calculated concentration (ng ml^{-1})[a]	RSD (%)	Accuracy (%)[b]
1.0	1.0	3.1	97.0
2.0	2.1	4.4	105.5
5.0	5.1	4.6	102.0
10.0	9.3	3.3	93.5
20.0	19.9	1.3	99.3
50.0	51.4	2.2	102.8
100.0	96.1	0.6	96.1
200.0	189.7	1.0	94.9
500.0	493.8	0.9	98.8
1000.0	984.4	0.4	98.4

[a] Expressed as RSD; $n=5$.
[b] Expressed as [(mean calculated concentration)/(nominal concentration)]×100.

Table 4
Inter-day precision[a] and accuracy of the assay of L-770,644 in dog plasma

Nominal concentration (ng ml^{-1})	Calculated concentration (ng ml^{-1})[a]	RSD (%)	Accuracy (%)[b]
1	0.9	14.7	85.2
5	5.3	7.2	106.3
10	9.5	8.7	95.4
20	19.9	6.7	99.4
50	53.6	5.0	107.2
100	105.2	4.6	105.2
200	205.6	7.6	102.8
500	523.9	4.8	104.8
1000	982.5	2.2	98.3

[a] Expressed as RSD; $n=6$.
[b] Expressed as [(mean calculated concentration)/(nominal concentration)]×100.

the MS–MS product ion spectra were used to selectively monitor L-770,644 and the internal standard (Fig. 4) in the LC effluent.

Assay intra-day precision and accuracy were determined by replicate analyses of control plasma containing known concentrations of L-770,644. The precision was less than 5% over the range of the assay (Table 3) and the accuracy was adequate at all concentrations. Inter-day precision (Table 4) was calculated from six data sets acquired over an 8-month period. Precision was less than 10% for all concentrations except at 1.0 ng/ml, which with a RSD of 15% and an accuracy of 85% was still sufficient for the purpose of the assay.

4. Conclusions

This HPLC–APCI-MS–MS method has been used over the past 6 years for the PK screening of over 1000 compounds. It has been applied to pooled samples, cassette dosing and single dose experiments. A mobile phase of 20 m*M* NH_4OAc with 0.4% TFA in 60% acetonitrile/water on a 4.6×50 mm cyano phase column produces total run times of less than 2 min for all of the compounds tested. This allows for the rapid optimization of assay conditions. The mass spectrometer can be optimized and the samples processed and analyzed, all in the same day. Compounds of different structural type can be ana-

lyzed using the same assay conditions. Multiple assays can be run consecutively without changing the column or mobile phase between assays, permitting the most efficient allocation of instrument time. Rapid PK screening with this method has helped to direct the synthetic efforts of medicinal chemists toward the production of compounds with desirable pharmacokinetic properties.

References

[1] T.R. Covey, E.D. Lee, J.D. Henion, Anal. Chem. 58 (1986) 2453.
[2] J.D. Gilbert, T.V. Olah, A. Barrish, T.F. Greber, Biol. Mass Spectrom. 21 (1992) 341.
[3] J.D. Gilbert, T.V. Olah, D.A. McLoughlin, ACS Symp. Ser. 619 (1996) 330.
[4] H. Founda, M. Nocerini, R. Schneider, C. Gedutis, J. Am. Soc. Mass Spectrom. 2 (1991) 164.
[5] J. Ayrton, R. Plumb, W.J. Leavens, D. Mallett, M. Dickins, G.J. Dear, Rapid Commun. Mass Spectrom. 12 (1998) 217.
[6] T. Yasuda, T. Mitsuko, K. Iba, J. Mass Spectrom. 31 (1996) 879.
[7] R.K. Gilpin, L.A. Pachla, Anal. Chem. 69 (1997) 145.
[8] D.M. Garcia, S.K. Huang, W.F. Stansbury, J. Am. Soc. Mass Spectrom. 7 (1996) 59.
[9] D.L. Buhrman, P.I. Price, P.J. Rudewicz, J. Am. Soc. Mass Spectrom. 7 (1996) 1099.
[10] M.G. Ikonomou, A.T. Blades, P. Kebarle, Anal. Chem. 62 (1990) 957.
[11] T. Ten-Yang, J. Charle, R.D. Voyksner, J. Am. Soc. Mass Spectrom. 7 (1996) 1106.
[12] W.H. Schaefer, F. Dixon Jr., J. Am. Soc. Mass Spectrom. 7 (1996) 1059.
[13] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (1998) 882.
[14] Y. Wu, J. Zhao, J. Henion, W.A. Korfmacher, A.P. Lapiguera, C. Lin, J. Mass Spectrom. 32 (1997) 379.
[15] T.L. Shih, M.R. Candelore, M.A. Cascieri, S.L. Chiu, L.F. Colwell Jr., L. Deng, W.P. Feeney, M.J. Forrest, G.J. Hom, D.E. MacIntyre, R.R. Miller, R.A. Sterans, C.D. Strader, L. Tota, M.J. Wyratt, M.H. Fisher, A.E. Weber, Bioorg. Med. Chem. Lett. 9 (1999) 1251.